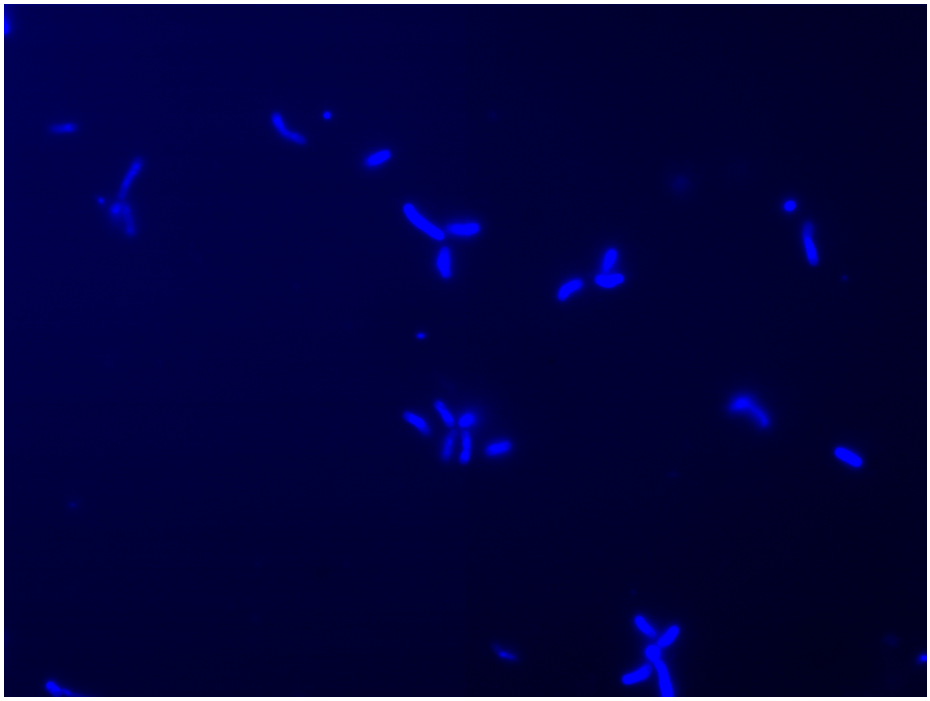
UNIVERSIDAD AUTÓNOMA DE MADRID
FACULTAD DE CIENCIAS
DEPARTAMENTO INTERUNIVERSITARIO DE ECOLOGÍA

EL ACUÍFERO DE DOÑANA COMO UN SISTEMA ECOLÓGICO: ESTRUCTURA Y FUNCIÓN DE SUS COMUNIDADES MICROBIANAS



TESIS DOCTORAL

Sergio Velasco Ayuso

Madrid, 2010

UNIVERSIDAD AUTÓNOMA DE MADRID
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EL ACUÍFERO DE DOÑANA COMO UN SISTEMA ECOLÓGICO: ESTRUCTURA Y FUNCIÓN DE SUS COMUNIDADES MICROBIANAS

Sergio Velasco Ayuso

Memoria presentada para optar al grado de
Doctor en Ciencias Biológicas

Trabajo dirigido por la Dra. María del Carmen Guerrero Sánchez y la Dra. Ana Isabel López Archilla, profesoras titulares del Departamento Interuniversitario de Ecología de la Universidad Autónoma de Madrid

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La tesis doctoral que lleva por título

EL ACUÍFERO DE DOÑANA COMO UN SISTEMA ECOLÓGICO: ESTRUCTURA Y FUNCIÓN DE SUS COMUNIDADES MICROBIANAS

de la que es autor Sergio Velasco Ayuso, ha sido realizada en el Departamento Interuniversitario de Ecología de la Universidad Autónoma de Madrid bajo la dirección de la Dra. María del Carmen Guerrero Sánchez y la Dra. Ana Isabel López Archilla.

Dra. María del Carmen Guerrero Sánchez Dra. Ana Isabel López Archilla

Sergio Velasco Ayuso

A María de la Cruz y Guillermo,
mis padres

Cualquier gran viaje comienza con un pequeño paso
Lao Tsé

AGRADECIMIENTOS

El documento que tienes ahora entre manos es el resultado de un largo trabajo que comenzó a finales de un verano, hace exactamente siete años. Recuerdo perfectamente que todo empezó con una llamada telefónica en la que Carmen Guerrero me decía que sí, que el proyecto iba a ser aprobado y que iba a haber un dinero destinado a un becario. Por tanto, es menester empezar agradeciendo a Carmen la confianza que depositó en mí para llevar las riendas del proyecto y, de paso, realizar una tesis doctoral. Es evidente que ésta no hubiera llegado a buen puerto sin los consejos y la ayuda de Ana Isabel López; muchas gracias Anabel por ellos, y por tu compañía en Doñana, claro. Y por supuesto, mucho tengo que agradecer también a Carlos Montes, y a su visión *holística* del mundo que nos rodea; muchas gracias Carlos por todas tus buenas ideas, no siempre fáciles de aplicar e interpretar, por cierto, pero muy interesantes en cualquier caso.

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No me puedo olvidar de la ayuda recibida por parte de Marisol Manzano, de la Universidad Politécnica de Cartagena. La visita que me llevó hasta allí durante unos días justo antes de las navidades de 2004 fue no solamente instructiva sino también esperanzadora tras *mis primeras aventuras* en Doñana. Ni tampoco de la de Jesús Pozo y todo su estupendo equipo de trabajo en la Universidad del País Vasco; ellos me ayudaron a introducirme en el mundo de las enzimas.

En mi memoria ha quedado grabado para siempre el viaje que me llevó a principios de 2006, durante cuatro meses, a Estados Unidos. Fue un viaje que me ayudó mucho en la elaboración de esta tesis doctoral, pero sobre todo me aportó mucho desde el punto de vista personal. En Florida, David L. Balkwill me acogió muy amablemente en su laboratorio de microbiología. Tengo tantas cosas que agradecerle que no sé muy bien por dónde empezar. Muchas gracias por los consejos, muchas gracias por ayudarme con el inglés y muchas gracias por saber transmitir tan bien todo lo que sabes. Por supuesto no me olvido de la gente de su laboratorio, con los que compartí unas cuantas horas; especialmente agradezco la compañía, el cariño y toda la ayuda brindada por parte de April y de Mindy; sin vosotras mi estancia hubiera sido bastante más complicada. Allí conocí también a unos maravillosos húngaros con los que hice piña y con los que pasé muy buenos ratos; Gyula, Zsuzsa, no olvidaré nunca esos partidos de *rackcetball*…

Mención aparte y muy, muy especial merece la ayuda, el apoyo y el ánimo para continuar hasta el final que me he prestado Robert L. Sinsabaugh, de la Universidad de Nuevo México. Sin ni

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Durante los últimos años he estado trabajando en el Centro de Estudios Hidrográficos del CEDEX. Y por supuesto tengo que agradecer de igual manera la ayuda que me han prestado, sobre todo moral, algunas personas aquí. Muchas gracias a Manolo, a Enrique, a Guille, a Juan, a Raúl, a Carlos, a Caridad, a Delia, a María Verdugo, a Yasmina, a Belén, a Laura Conde, a Raquel, a Toni, a Adela, a Marta, a Felipe, a Nacho, a Covita, a María Peg, a María Elena, a Laura Hernández, etc. Mención aparte merece mi compañera de despacho, Elena, que es la que más directamente ha sufrido en el CEDEX mis problemas con la tesis; muchas gracias por toda tu ayuda Elenita.

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Y ya por último no me queda nada más que decir que si hay algo que me ha hecho llegar hasta el final con este trabajo es Doñana, un paraje único en el mundo que te enamora y te atrapa de tal forma que resulta difícil de explicar si no estás allí y lo ves con tus propios ojos. Y es que, como una vez me dijeron, *Doñana hay que vivirlo*…

Seguro que me he dejado a alguien por el camino, pero no me lo tengas en cuenta si eres tú uno o una de ellos/as y siéntete agradecido/a de la misma manera que los demás.

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CAPÍTULO 1. Introducción general

1. INTRODUCCIÓN GENERAL

JUSTIFICACIÓN Y ANTECEDENTES

Los acuíferos han sido históricamente muy importantes porque han constituido la base para el desarrollo socioeconómico de muchas civilizaciones debido a que proveen numerosos bienes y servicios, ligados fundamentalmente a su concepción como formaciones geológicas capaces de almacenar y transmitir agua en grandes cantidades (Balkwill y Ghiorse, 1985). Debido a ello, los acuíferos han sido tradicionalmente estudiados desde un punto de vista exclusivamente hidrogeológico, siendo considerados como simples embalses subterráneos (Balkwill y Ghiorse, 1985). En este sentido, de entre todos los ecosistemas presentes en la superficie terrestre o bajo la corteza de nuestro planeta, los aspectos biológicos y ecológicos de las aguas subterráneas en particular y de los acuíferos en general son los menos explorados y conocidos (Cullimore, 2008). Este hecho es realmente sorprendente si se tiene en cuenta que más del 97% de todo el agua planetaria no marina y que no está helada se encuentra formando parte de los acuíferos (Gibert *et al*., 1994), pero es que hasta hace poco más de treinta años se pensaba que la biosfera no se extendía más allá de unos pocos centímetros por debajo de la rizosfera (Griebler y Lueders, 2009). Si el afán de conocimiento, junto con la curiosidad, ha sido la principal motivación para estudiar la biosfera desde un punto de vista científico, dada la poca accesibilidad de los acuíferos y la ausencia de animales y de plantas en ellos, objetos de estudio preferentes en otros sistemas, no es entonces sorprendente que el estudio de las características biológicas y ecológicas de los sistemas acuíferos presente un considerable retraso con respecto al de otros sistemas (Madsen y Ghiorse, 1993).

Sin embargo, la aproximación al estudio de los sistemas acuíferos ha cambiado radicalmente en las últimas tres décadas, pasando de ser exclusivamente hidrogeológica a poseer una perspectiva bastante más ecológica (Baker *et al*., 2000). Como consecuencia, actualmente, desde el punto de vista de la ecología, los acuíferos son considerados como un conjunto heterogéneo de microhábitats

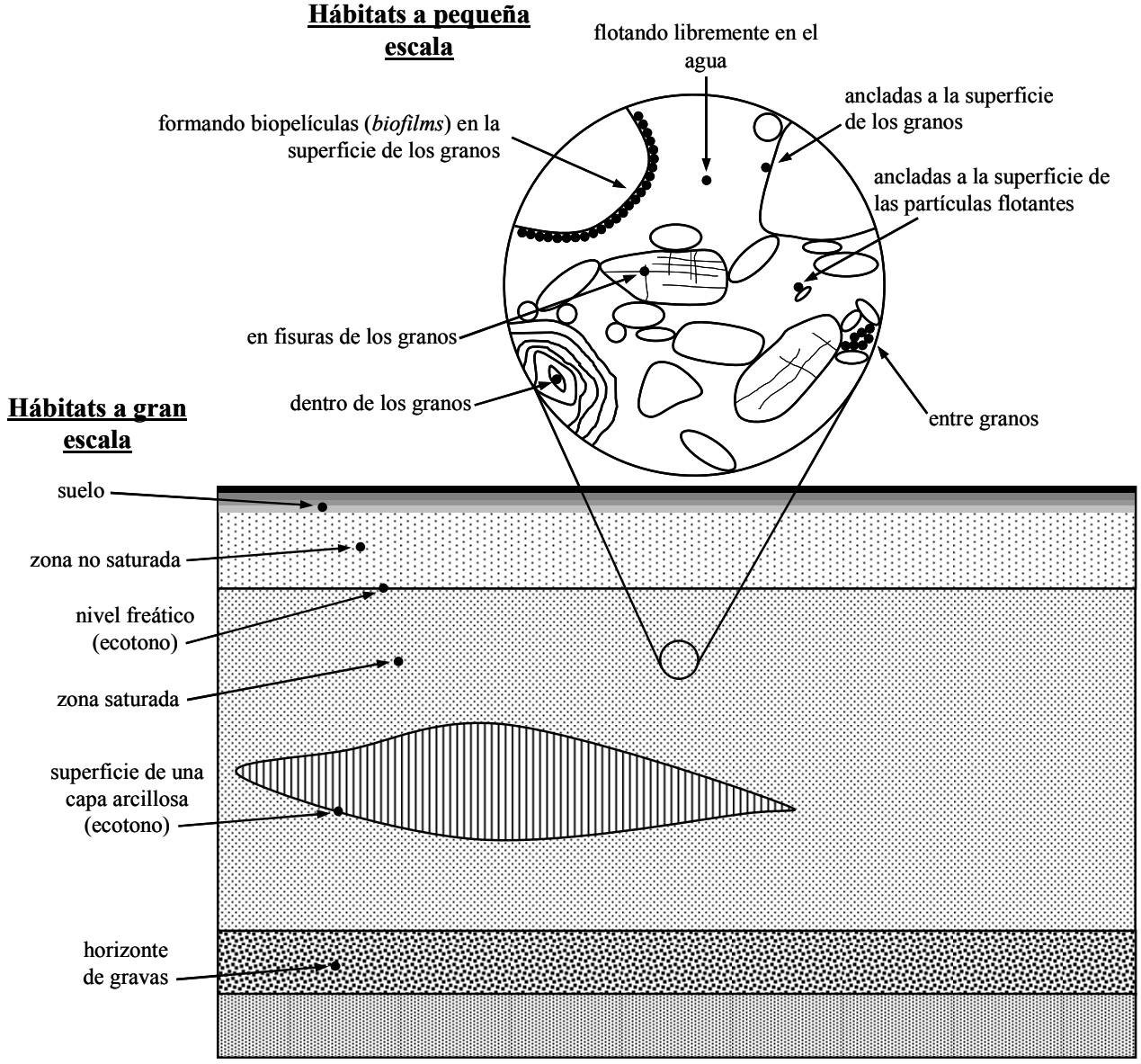

Figura 1.1 Esquema que muestra el conjunto heterogéneo de microhábitats y macrohábitats que permiten la existencia de comunidades microbianas en acuíferos arenosos (Modificado de Goldscheider *et al.*, 2006).

y macrohábitats que proporcionan una gran cantidad de condiciones diferentes para el desarrollo de las comunidades biológicas, fundamentalmente microbianas (Goldscheider *et al*., 2006) (Figura 1.1). Desde un punto de vista ecológico, los acuíferos, al menos los más someros, deben ser reconocidos como sistemas abiertos que interaccionan e intercambian permanentemente materia y energía con otros sistemas, tanto acuáticos como terrestres, que se localizan en sus proximidades y que pueden ser superficiales o subterráneos. El estudio de estas interacciones a diferentes escalas espaciales y temporales resulta fundamental para conocer el papel ecológico que juegan las comunidades microbianas en los acuíferos, así como la influencia que la actividad de las mismas puede tener sobre los procesos ecológicos que tienen lugar en los sistemas con los que los acuíferos se relacionan (Hancock *et al*., 2005) (Figura 1.2).

La identificación de tales interacciones es una tarea muy compleja. Para lograr caracterizarlas, se requiere de estudios a diferentes escalas espaciales y con escalas temporales amplias. Este tipo de

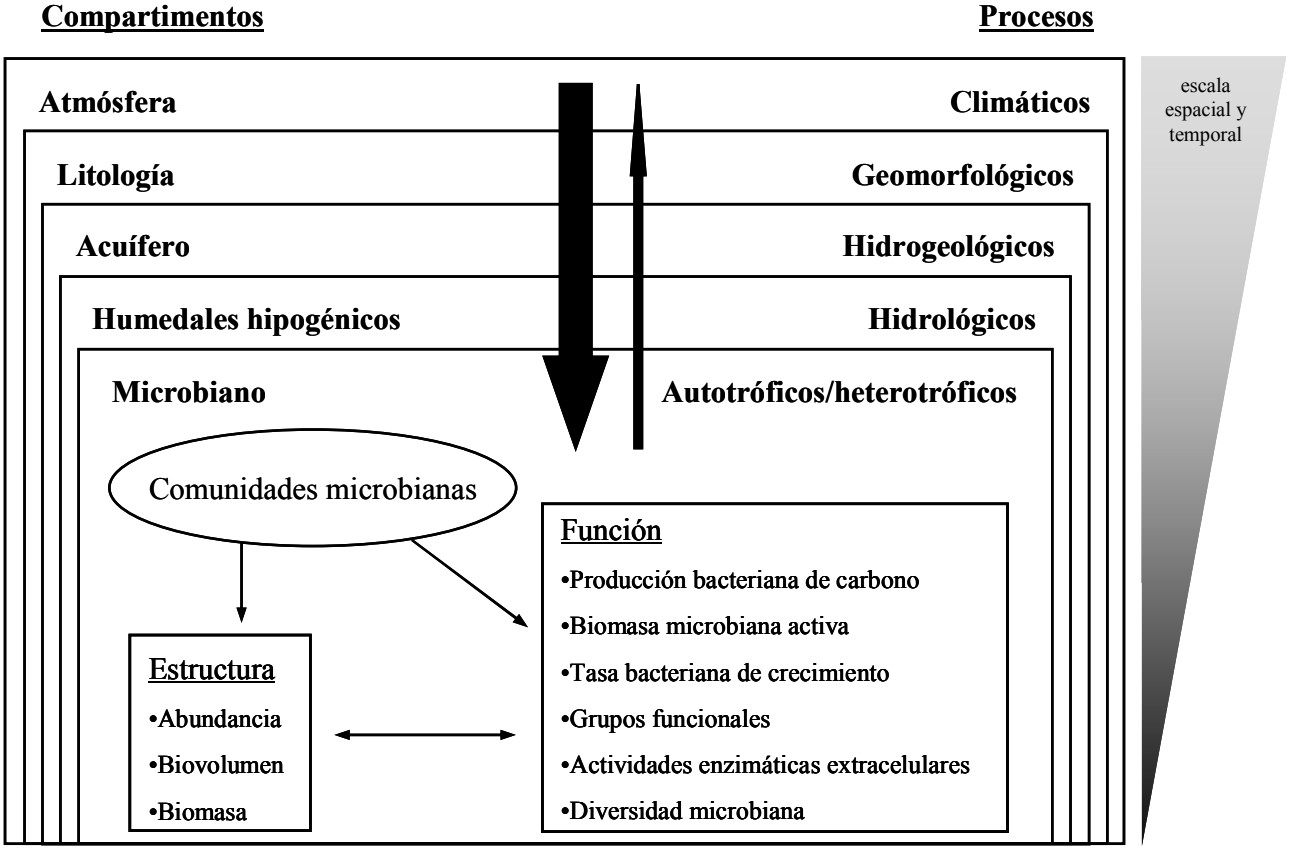

Figura 1.2 Modelo conceptual que muestra la acción, a diferentes escalas espaciales y temporales, de los procesos que controlan la estructura y la función de las comunidades microbianas en el acuífero de Doñana (Modificado de Montes *et al.*, 1998).

estudios no es habitual. Además, teniendo en cuenta que variables muy diversas pueden afectar de una u otra manera a estas interacciones, es muy importante que el estudio sea llevado a cabo en el marco de un equipo multidisciplinar de investigación. La realización de proyectos multidisciplinares conlleva un enorme esfuerzo económico y humano, ya que se basa en la generación de una importante cantidad de información experimental (Coleto, 2003). Por otro lado, es muy importante tener en mente que este tipo de proyectos multidisciplinares enfocados a conocer la estructura, el funcionamiento y la dinámica de los ecosistemas, solamente serán útiles para establecer esas interacciones cuando todas las aproximaciones compartan escalas espaciales y temporales (Musslewhite *et al*., 2003), y cuando exista una única coordinación general que fije una metodología y unos objetivos comunes (Coleto, 2003).

En el año 1997 comenzó su andadura el proyecto multidisciplinar MADRE I (*Management of Doñana´s Aquatic Recources and Ecosystems*), financiado por la Comisión Interministerial de Ciencia y Tecnología (CICYT), cuyo objetivo principal fue el de estudiar, mediante técnicas de evaluación funcional de humedales, las interacciones entre los diferentes sistemas acuáticos de los mantos eólicos y flecha litoral de El Abalario-Doñana (suroeste de España) desde una perspectiva ecosistémica, jerárquica y multidisciplinar (Coleto, 2003). El desarrollo de ese proyecto multidisciplinar supuso una vía para obtener las bases científicas de apoyo a la gestión integral de los recursos hídricos, muy necesarias en un territorio en donde el componente clave del funcionamiento general es la propia agua, tanto superficial como subterránea (Coleto, 2003), ya que

no se puede concebir la gestión integral de los recursos hídricos de un territorio sin caracterizar sus ecosistemas convenientemente (Montes *et al*., 1998). De este proyecto MADRE I surgieron, entre muchos otros trabajos, dos tesis doctorales (Álvarez, 2002; Coleto, 2003) que han servido como base para la realización del presente estudio. Los resultados más relevantes de ambas tesis doctorales demostraron, por un lado, la importancia de las comunidades microbianas de los sedimentos de los humedales hipogénicos de Doñana en procesos ecológicos claves para el correcto funcionamiento de los sistemas acuáticos (Álvarez, 2002) y, por otro, la importancia de considerar a las aguas subterráneas y a las aguas superficiales como integrantes de un único sistema, que es dinámico y que controla la variabilidad espaciotemporal de varios procesos ecológicos clave en la comarca de Doñana (Coleto, 2003). Este sistema, que incluye también a las aguas salobres de las marismas de Doñana, se ha denominado *hidroecosistema* (Montes *et al*., 1998).

El presente trabajo de tesis doctoral se enmarca dentro de otro proyecto multidisciplinar denominado MADRE II, que comenzó en 2002. Al igual que el anterior proyecto, éste ha sido financiado por la CICYT y su objetivo principal ha sido el de dar continuación a los resultados obtenidos por el proyecto MADRE I, centrándose en los aspectos no abordados directamente por éste, pero que fueron, sin embargo, parcialmente delineados (Álvarez, 2002; Coleto, 2003). En este sentido, el proyecto MADRE II, cuyo título completo es *Gestión de Recursos Hídricos y Conservación de los Humedales Hipogénicos del Manto Eólico Litoral de Doñana*, ha tenido como ambicioso objetivo el estudio de las aguas subterráneas de Doñana desde una perspectiva ecológica, multidisciplinar, jerárquica y holística (Figura 1.2), enfocado, en último término, hacia la gestión integral de los recursos hídricos de la comarca.

El trabajo que conforma la presente tesis doctoral se centra en todos aquellos aspectos relativos a la ecología de las comunidades microbianas del sistema acuífero de Doñana. Este estudio pretende abordar cuestiones relacionadas con la dinámica espaciotemporal de la estrucura y de la función de las comunidades microbianas en las aguas subterráneas de Doñana (Figura 1.2) y, de igual modo, aportar datos que permitan establecer la conectividad biológica entre dos de los compartimentos acuáticos de Doñana, aguas superficiales y aguas subterráneas, teniendo en cuenta que estas últimas no constituyen un compartimento cerrado en el gran ecosistema fluviolitoral de Doñana (GED), sino que forman parte del *hidroecosistema* anteriormente mencionado, en el que se incluyen también las aguas salobres de las marismas (Montes *et al*., 1998). Para ello, primero es clave determinar la estructura de estas comunidades microbianas en términos de abundancia, biovolumen y biomasa, variables básicas que pueden dar una idea general de la actividad potencial de una comunidad y de su valor como recurso alimenticio para niveles tróficos superiores. En segundo lugar, es fundamental determinar hasta qué punto y en qué sentido estas comunidades microbianas son funcionales. Entre las diversas variables indicativas de la actividad y la diversidad funcional de la microbiota del acuífero se han estimado valores sobre producción bacteriana de carbono, biomasa microbiana activa, tasa bacteriana de crecimiento, grupos funcionales, actividades enzimáticas extracelulares (AEE) y diversidad microbiana. Todas estas variables son indicativas del estado fisiológico de las comunidades microbianas y de sus productividades e implicaciones en los diferentes ciclos biogeoquímicos del ecosistema.

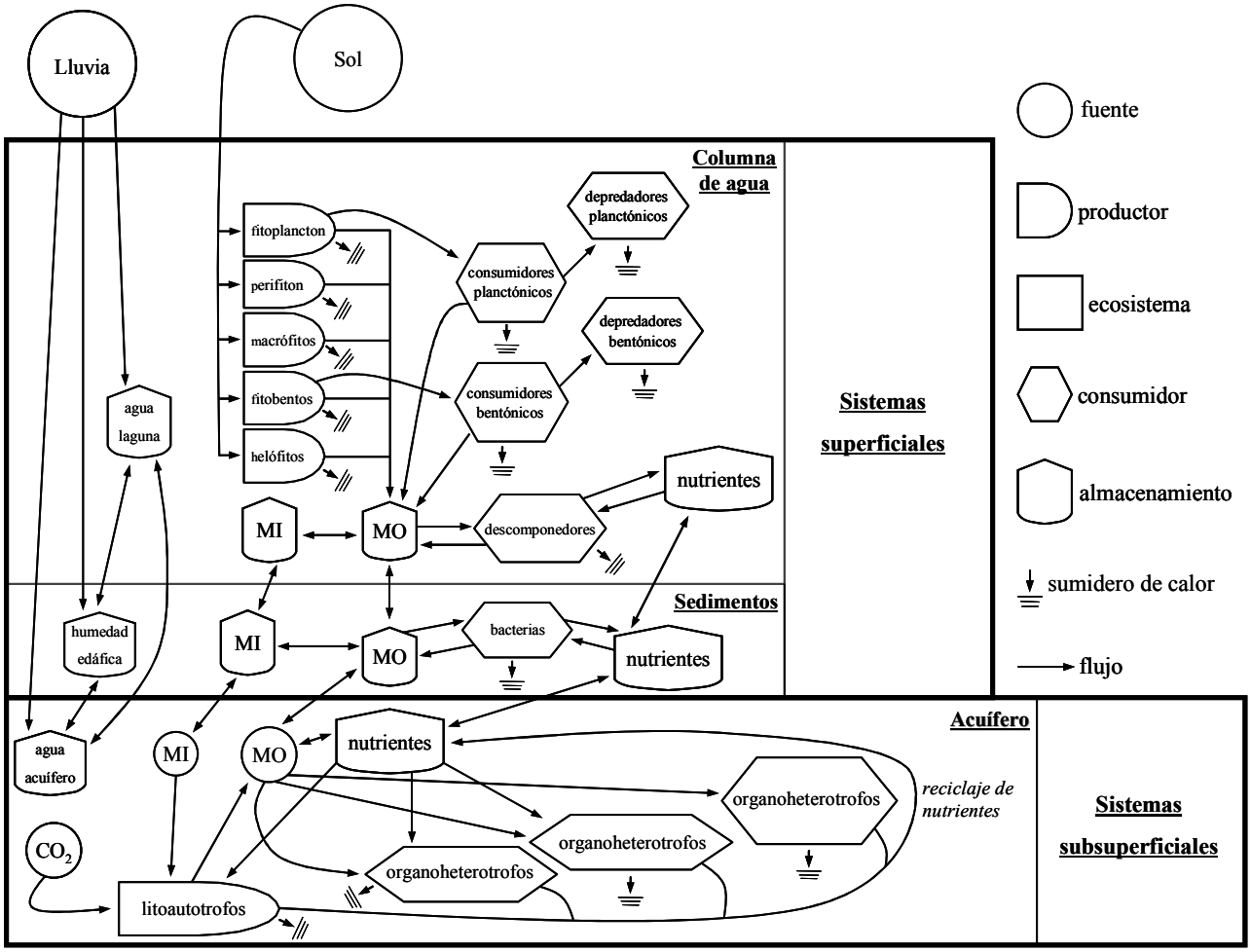

Figura 1.3 Esquema conceptual, basado en la nomenclatura de Odum y Barrett (2001), de los principales flujos de materia y energía entre diferentes compartimentos del *hidroecosistema* de Doñana. El esquema del compartimento de los sistemas superficiales fue desarrollado bajo el proyecto MADRE I, mientras que el de los sistemas subsuperficiales se ha planteado bajo el proyecto MADRE II.

El trabajo que se presenta a continuación es pionero en España y particularmente en Doñana, donde nunca antes se había abordado el estudio del sistema acuífero desde una perspectiva ecológica. Sin embargo, desde un punto de vista hidrogeológico, este acuífero es uno de los que mejor se conocen en España (Custodio *et al*., 2009). Este hecho, unido a que Doñana constituye un área emblemática en la conservación y gestión de espacios naturales en la península Ibérica, convierte a la zona en un escenario ideal para un análisis funcional como el que aquí se plantea (Álvarez, 2002; Coleto, 2003). Además, el acuífero de Doñana constituye el elemento vertebrador que mantiene los procesos ecológicos de todos los ecosistemas de Doñana que se relacionan con él, tanto terrestres como acuáticos. En este sentido, la importancia ecológica de los humedales de Doñana es enorme (Coleto, 2003); la mayoría de estos humedales se encuentra en los mantos eólicos litorales/flecha litoral, en donde hay inventariadas unas 680 formaciones palustres que están alimentadas en casi un 95% por aguas subterráneas (Coleto, 2003). Este complejo palustre no es solamente el más importante de España sino también uno de los más importantes de Europa. Actualmente, el sistema acuífero está amenazado por diversas actividades de origen antrópico, como las extracciones de agua para usos agrícolas y turísticos o la contaminación por compuestos procedentes de la agricultura. De ahí la apremiante necesidad de establecer las bases científicas que

permitan mantener su integridad ecológica, entendida como el conjunto de procesos físicos, químicos y biológicos que definen la organización, el funcionamiento y la dinámica de un ecosistema (Montes *et al.*, 1998). Sin embargo, esta integridad solamente se podrá alcanzar si se tienen en cuenta todos los elementos que forman parte del *hidroecosistema* de Doñana, entre los que las comunidades microbianas del acuífero parecen haberse perfilado como muy importantes.

La Figura 1.3 muestra el modelo conceptual basado en la nomenclatura de Odum y Barrett (2001) que ha servido de guía para el desarrollo de esta tesis doctoral. Es una figura que amplía la visión que el anterior proyecto MADRE I tenía sobre los sistemas acuáticos de Doñana, incluyendo ahora el acuífero como un sistema ecológico más que forma parte del *hidroecosistema* de Doñana. Como quedó reflejado en las tesis doctorales de S. Álvarez (2002; Figura 1.1, página 8) y M.C. Coleto (2003; Figura 1.1, página 7), el proyecto MADRE I consideraba que el acuífero era una especie de caja negra en la que se llevaban a cabo una serie de procesos ecológicos que, aunque aparentemente muy importantes para comprender el funcionamiento global de este *hidroecosistema*, no fueron estudiados, aunque sí parcialmente delineados (Álvarez, 2002). Con la finalidad de estudiar el acuífero de Doñana desde una perspectiva ecológica, para el proyecto MADRE II se diseñó un protocolo de muestreo con un doble propósito. En primer lugar, y con el objetivo de conocer las características globales de las comunidades microbianas del sistema acuífero, se planteó un muestreo extensivo que abarcó 30 piezómetros en un área de unos 100 km² y con profundidades comprendidas entre los 2 y los 72 metros bajo la superficie del terreno. Por otra parte, para tratar de describir con mayor precisión las relaciones entre el sistema acuífero y los humedales hipogénicos, se llevó a cabo un muestreo intensivo estacional, durante dos ciclos hidrológicos, en 13 piezómetros localizados en los alrededores de cuatro lagunas intensamente estudiadas en el proyecto MADRE I.

La concepción del acuífero como un sistema ecológico que interacciona permanentemente a diferentes escalas espaciales y temporales con otros sistemas presentes en Doñana, tanto terrestres como acuáticos, con los que intercambia materiales y energía, es la clave para entender todo el trabajo llevado a cabo en esta tesis doctoral.

OBJETIVO E HIPÓTESIS GENERAL

<u>*Objetivo general*</u>: conocer y describir el papel ecológico de las comunidades microbianas del acuífero de Doñana a través de la determinación de variables estructurales (abundancia bacteriana, biomasa celular y biomasa bacteriana) y de variables funcionales (grupos funcionales, biomasa microbiana activa, producción bacteriana de carbono, tasa bacteriana de crecimiento, actividades enzimáticas extracelulares, diversidad microbiana). Para ello se realizará una aproximación intensiva en varios piezómetros localizados en los alrededores de cuatro humedales que fueron estudiados durante el proyecto MADRE I y una aproximación extensiva que engloba un área de unos 100 km^2, considerando al acuífero como un ecosistema y no simplemente como un embalse de agua subterránea.

<u>*Hipótesis general*</u>: las comunidades microbianas del acuífero de Doñana serán abundantes y activas, aunque relativamente estables debido a las pequeñas oscilaciones que suelen presentar algunas

variables ambientales en los sistemas subterráneos, tales como la temperatura o el oxigeno disuelto. Las variables que determinan tanto la estructura como la función de estas comunidades dependerán en gran medida de las relaciones entre las aguas subterráneas y las aguas superficiales, al menos en la parte más somera de este sistema acuífero, tal y como se ha demostrado con anterioridad en otros acuíferos (Sophocleous, 2002).

OBJETIVOS PARTICULARES

Objetivo 1: estudiar la dinámica espaciotemporal de la estructura de las comunidades microbianas del acuífero de Doñana mediante la determinación de la abundancia bacteriana, la biomasa celular y la biomasa bacteriana, a través de una aproximación extensiva que englobe información de un área de unos 100 km² y de profundidades comprendidas entre los 2 y los 72 metros bajo la superficie del terreno.

Objetivo 2: determinar la función de las comunidades microbianas del acuífero de Doñana mediante el uso de ensayos en tubos BART™ (*Biological Activity Reaction Tests*) y la estimación de la biomasa microbiana activa, la producción bacteriana de carbono y la eficiencia bacteriana de crecimiento, a través de una aproximación extensiva que englobe información de un área de unos 100 km² y de profundidades comprendidas entre los 2 y los 72 metros bajo la superficie del terreno.

Objetivo 3: describir la influencia de determinados factores ambientales, como la profundidad, el tamaño de grano o la temperatura, entre otros, sobre la estructura y la función de las comunidades microbianas del acuífero de Doñana.

Objetivo 4: analizar la dinámica espaciotemporal de las actividades enzimáticas extracelulares de las comunidades microbianas del acuífero de Doñana (β-D-glucosidasa, leucina aminopeptidasa, fosfatasa alcalina y fenol oxidasa), y de las variables que las controlan, para determinar la participación de estas comunidades en los ciclos biogeoquímicos.

Objetivo 5: describir y estudiar las variables que controlan espaciotemporalmente la distribución de la estructura y de la función de las comunidades microbianas en la parte más somera del acuífero de Doñana mediante una aproximación intensiva en 13 piezómetros localizados en los alrededores de cuatro humedales muy productivos situados sobre los mantos eólicos.

Objetivo 6: analizar, junto con los resultados obtenidos en el objetivo anterior, las relaciones entre las comunidades microbianas localizadas en la parte más somera del acuífero y los procesos ecológicos que se llevan a cabo en los sistemas acuáticos superficiales mediante una aproximación intensiva, considerando que el acuífero no es un compartimento aislado en el *hidroecosistema* de Doñana.

Objetivo 7: estudiar la diversidad de las comunidades microbianas del acuífero de Doñana mediante técnicas de amplificación, clonación y secuenciación de ARNr 16S con el fin de caracterizar los diferentes mecanismos energéticos que se llevan a cabo en las aguas subterráneas y definir la implicación de estas comunidades microbianas en los ciclos biogeoquímicos.

ESTRUCTURA DE LA MEMORIA

Esta memoria consta de nueve capítulos cuyos contenidos se exponen a continuación.

El capítulo 1 (Introducción general) justifica y enmarca el presente trabajo en estudios previos, exponiendo tanto el objetivo y la hipótesis general como los objetivos particulares.

El capítulo 2 (Estado del conocimiento) constituye un breve resumen del estado del arte sobre la ecología de las aguas subterráneas, particularmente centrado en las comunidades microbianas

El capítulo 3 (La estructura de las comunidades microbianas del acuífero de Doñana I: una aproximación intensiva) describe la estructura de las comunidades microbianas del acuífero de Doñana en términos de abundancia bacteriana, biomasa celular y biomasa bacteriana a través del estudio intensivo de las aguas subterráneas localizadas en los alrededores de cuatro humedales muy productivos durante dos ciclos hidrológicos. Asimismo, también analiza la presencia de grupos funcionales de bacterias (bacterias del hierro, bacterias sulfatorreductoras, bacterias desnitrificantes, bacterias nitrificantes). Los resultados de este estudio han sido publicados en la revista *Hydrogeology Journal*, 17, 767-780 (2009).

El capítulo 4 (La estructura de las comunidades microbianas del acuífero de Doñana II: una aproximación extensiva) aborda un estudio paralelo al del capítulo 3, ampliando la escala espacial a unos 100 km^2 y manteniendo la misma escala temporal de campañas estacionales de muestreo a lo largo de dos ciclos hidrológicos. Los resultados de este estudio han sido publicados en la revista *Geobiology*, 7, 66-81 (2009).

El capítulo 5 (La función de las comunidades microbianas del acuífero de Doñana I: producción bacteriana de carbono) se centra en la descripción de las actividades funcionales de las comunidades microbianas del acuífero de Doñana mediante el estudio de la producción bacteriana de carbono y otras variables relacionadas con ésta, como la biomasa microbiana activa y la tasa bacteriana de crecimiento. Los resultados de este estudio se han publicado en la revista *Geomicrobiology Journal*, 27, 409-423 (2010).

El capítulo 6 (La función de las comunidades microbianas del acuífero de Doñana II: actividades enzimáticas extracelulares) comprende un estudio sobre las actividades enzimáticas extracelulares de las comunidades microbianas del acuífero de Doñana y su implicación en los ciclos biogeoquímicos, presentando datos sobre tasas enzimáticas de β-D-glucosidasa, leucina aminopeptidasa, fosfatasa alcalina y fenol oxidasa. Los resultados de este estudio se han enviado a la revista *Aquatic Microbial Ecology*.

El capítulo 7 (La diversidad de las comunidades microbianas del acuífero de Doñana) presenta un estudio sobre la diversidad microbiana del acuífero de Doñana abordada mediante técnicas de amplificación, clonación y secuenciación de genes de ARNr 16S en muestras naturales de dos piezómetros, uno somero y otro profundo. Los resultados de este trabajo fueron publicados en la revista *Aquatic Microbial Ecology*, 47, 123-139 (2007).

El capítulo 8 (Discusión general) presenta un modelo de funcionamiento general del acuífero de Doñana entendido como ecosistema, basado en la información obtenida a lo largo del estudio. Además, se muestra la conectividad biológica existente entre los compartimentos del *hidroecosistema*. La importancia ecológica de las comunidades microbianas se discute en términos de la diversidad de mecanismos energéticos detectados en el acuífero y su implicación en los diferentes ciclos biogeoquímicos.

El capítulo 9 (Conclusiones) muestra las principales conclusiones derivadas de esta tesis doctoral.

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CAPÍTULO 2. Estado del conocimiento

2. ESTADO DEL CONOCIMIENTO

La ecología microbiana es una disciplina que ha sufrido un gran avance en las últimas décadas debido en parte a la mejora de las técnicas disponibles para realizar medidas *in situ* de las principales variables que determinan tanto la estructura como la función de las comunidades microbianas en los sistemas naturales (Atlas y Bartha, 1997). En un sentido amplio, las nuevas técnicas han posibilitado la descripción de las comunidades microbianas como dinámicas y metabólicamente muy activas, permitiendo además considerarlas como el componente biológico principal en los flujos de materia y de energía en cualquier ecosistema (Chróst y Overbeck, 1994).

En los ecosistemas acuáticos, el desarrollo de la ecología microbiana ha sido muy asimétrico, centrándose la mayor parte de los estudios en los sistemas epicontinentales y en los sistemas marinos. Los sistemas acuíferos, en cambio, han sido estudiados mayoritariamente desde un punto de vista hidrogeológico. En este sentido, el anglicismo *groundwater* se refiere única y exclusivamente al agua cuya extracción es sencilla cuando se acumula en sustratos saturados y altamente permeables conocidos como acuíferos (Custodio y Llamas, 1983). Sin embargo, a lo largo de los últimos 30 años, el estudio de los acuíferos y de sus comunidades microbianas se ha abordado desde una perspectiva más ecológica (Griebler y Lueders, 2009). Además, dado que el agua es imprescindible para el desarrollo de la vida, el estudio de las comunidades microbianas de las aguas subterráneas no solamente se ha circunscrito a los acuíferos sino que se ha extendido también hacia otras formas de agua, como el agua capilar o el agua de las zonas no saturadas de un acuífero libre (Madsen y Ghiorse, 1993) (Figura 1.1).

La ecología de las aguas subterráneas se define como el estudio de las interacciones entre los organismos que se desarrollan en ellas y las condiciones ambientales que existen en este tipo de ecosistemas (Danielopol, 1994). En este sentido, actualmente se pueden diferenciar distintos campos dentro de la ecología de las aguas subterráneas, como la bioespeleología, que ha derivado

en la actualidad hacia la ecología de los sistemas kársticos, la ecología de los sedimentos no consolidados, que ha dado origen a la freatobiología y a la ecología del medio hiporréico, y la ecología microbiana de los sistemas acuíferos, tanto someros como profundos (Hancock *et al.*, 2005). El objetivo principal del presente trabajo es el estudio de la ecología de las comunidades microbianas del acuífero de Doñana, por lo que la breve descripción sobre el estado del conocimiento que se ofrece a continuación deja de lado tanto a la ecología de los sistemas kársticos como a la freatobiología. No obstante, es importante tener en cuenta que esas tres disciplinas abordan conjuntamente el estudio de la ecología de las aguas subterráneas, compartiendo, por tanto, metodologías y aproximaciones (Gibert *et al.*, 2009).

El estudio de las comunidades microbianas de los sistemas acuáticos subterráneos es bastante reciente; de hecho, hasta la década de 1980 no aparecieron los primeros trabajos especializados sobre el tema (Ghiorse y Wilson, 1988; Madsen y Ghiorse, 1993; Gounot, 1996), debido fundamentalmente a que durante mucho tiempo se pensaba que la biosfera no se extendía más allá de los primeros horizontes de los suelos o de las capas de sedimentos húmedos de los ríos y de los lagos más profundos. De hecho, hasta hace no demasiado tiempo era ciertamente habitual escuchar entre los más prestigiosos ecólogos microbianos la idea de que las aguas subterráneas son "*esencialmente estériles*" y la de que sus condiciones para mantener poblaciones microbiológicas son "*escasas e insignificantes*" (Cullimore, 2008). Además, la tecnología para estudiar adecuadamente las capas profundas de la corteza terrestre desde un punto de vista microbiológico no fue desarrollada hasta finales de la década de 1970 y principios de la de 1980. Todo ello ha supuesto que el desconocimiento general de los ambientes subterráneos desde un punto de vista ecológico haya llegado casi hasta nuestros días (Gounot, 1996; Fredrickson y Balkwill, 2006; Cullimore, 2008).

Los primeros indicios sobre la existencia de microorganismos por debajo de los horizontes más someros de los suelos proceden de los estudios de algunos microbiólogos rusos del siglo XIX tan insignes como Sergei Winogradsky (Atlas y Bartha, 1997). Estos estudios, sin embargo, no constituían más que aproximaciones teóricas que trataban de resolver cuestiones sobre los sistemas naturales que se encontraban más allá de las posibilidades de la época, debido fundamentalmente a carencias técnicas. Sin embargo, la importancia que han tenido todas estas aproximaciones teóricas para el ulterior desarrollo de la ecología microbiana ha sido enorme.

Ya en el siglo XX, concretamente en 1919, un geólogo llamado Sherburne Rogers realizó un estudio para el Servicio Geológico de Estados Unidos (USGS) en el que postulaba que la oxidación de hidrocarburos en un acuífero somero de California estaba probablemente relacionada con la reducción de sulfatos y la producción de bicarbonatos. Rogers sugirió que las bacterias sulfatorreductoras podrían estar implicadas en las transformaciones químicas que él observaba. En ese momento, sin embargo, no había constatación clara de que los microorganismos pudiesen vivir en este tipo de ambientes. La confirmación de que éstos sí podían desarrollarse en los sistemas subterráneos se produjo gracias a los estudios pioneros de Edson S. Bastin y Frank E. Greer, de la Universidad de Chicago, sobre la presencia de bacterias reductoras de sulfato en aguas profundas

(Bastin *et al.*, 1926). El trabajo de Bastin y Greer puede considerarse como uno de los que constituyen la base de la actual ecología microbiana de acuíferos. Estos primeros estudios pioneros cayeron, sin embargo, en el olvido por parte de la comunidad científica, hasta que el Departamento de Defensa del gobierno de Estados Unidos puso en marcha un proyecto, a principios de la década de 1970, con el fin de construir silos subterráneos en los que almacenar ingentes cantidades de residuos radiactivos producidos durante la guerra fría (Fredrickson *et al.*, 2004). Teniendo en cuenta que los microorganismos, si están, pueden dañar peligrosamente la integridad estructural de estos silos (debido a fenómenos de biocorrosión, entre otros), parecía importante determinar y cuantificar la existencia de las comunidades microbianas en estos sistemas subsuperficiales. Los primeros resultados en cuanto a abundancia y actividad de microorganismos sorprendieron a los científicos, sobre todo a los más escépticos (Madsen y Bollag, 1989).

Más o menos al mismo tiempo, la presencia en las aguas subterráneas de ciertas sustancias potencialmente tóxicas, procedentes en su mayor parte de la industria y de la agricultura, dieron lugar a numerosos estudios enfocados a conocer y caracterizar las actividades de las comunidades microbianas de los acuíferos con el fin de emplearlas para mitigar los efectos nocivos que tienen para la salud humana estas sustancias. Este tipo de estudios tuvo un gran auge hace unos 30 años, debido principalmente a que el agua subterránea es fuente de agua dulce en muchos lugares. El rápido desarrollo de las técnicas que permitieron la caracterización de la estructura y de la función de las comunidades microbianas en los sistemas acuíferos con este fin permitió, no obstante, estudiar también estas comunidades desde un punto de vista ecológico, si bien es cierto que la mayor parte de los trabajos publicados durante las décadas de 1980 y 1990 acerca de las comunidades microbianas de acuíferos, se centraban en su papel como agentes biorremediadores en posibles escapes y/o filtraciones de una gran cantidad de agentes químicos (Haack y Bekins, 2000). Sin embargo, en los últimos años se ha podido observar un aumento muy significativo de trabajos que se centran en el estudio de estas comunidades microbianas desde un punto de vista más ecológico, es decir, considerándolas como partes integrantes del ecosistema en el que viven y en el que llevan a cabo sus actividades (Hancock *et al.*, 2005; Goldscheider *et al.*, 2006; Griebler y Lueders, 2009; Humphreys, 2009; Pronk *et al.*, 2009).

Desde la década de 1980, la presencia en acuíferos de comunidades microbianas funcionalmente activas y metabólica y taxonómicamente muy diversas ha quedado demostrada en una gran variedad de sistemas subsuperficiales (Balkwill *et al.*, 1989; Boivin-Jahns *et al.*, 1996; Pedersen *et al.*, 1996; Dojka *et al.*, 1998; Zhou *et al.*, 2004; Goldscheider *et al.*, 2006; Griebler y Lueders, 2009). Estas comunidades microbianas se componen principalmente de procariotas (bacterias y arqueas), hongos y protozoos (Madsen y Ghiorse, 1993), si bien los procariotas constituyen la forma de vida predominante en este tipo de sistemas, ya que se pueden desarrollar perfectamente bajo las múltiples condiciones que se suelen encontrar en estos ambientes (Balkwill y Ghiorse, 1985). Organismos más grandes (como por ejemplo microinvertebrados) aparecen en cuevas o en acuíferos cavernosos conectados con los sistemas superficiales (Gibert, 1988). Algunos organismos propios de medios hiporréicos (como algunos macroinvertebrados) pueden habitar a ciertas profundidades en los sedimentos de ríos o de lagos (Danielopol y Marmonier, 1992). La

aparente facilidad con la que se desplazan las bacterias, y los escasos protozoos que se alimentan de ellas, a través de los poros de los acuíferos, ya sea flotando libremente o ancladas a granos de sedimentos, ha quedado demostrada a través de diferentes experimentos, gracias a los que se puede deducir la interconexión amplia y robusta entre las capas húmedas de cierta profundidad y las más someras o superficiales (Harvey *et al.*, 1995; Balkwill *et al.*, 1998; Lehman *et al.*, 2001). No obstante, esta aparente facilidad de desplazamiento no es tal en ambientes más profundos. Entre los protozoos que pueden habitar los sistemas acuíferos se han identificado sobre todo flagelados; la presencia de ciliados parece ser más escasa (Gounot, 1996; Harvey *et al.*, 2002). Así mismo, conviene señalar que, en los acuíferos en los que se ha estudiado, la presencia de arqueas (Chyler *et al.*, 1998; López-Archilla *et al*., 2007) y de virus (Chapelle, 2001) suele ser muy importante.

Gracias a varios trabajos llevados a cabo en diferentes acuíferos de América (Stevens *et al.*, 1993; Raskin *et al.*, 1996; Fry *et al.*, 1997), Europa (Boissier *et al.*, 1996; Hess *et al.*, 1997; Kleikemper *et al.*, 2002; Tiirola *et al.*, 2002), Asia (Cho y Kim, 2000) o África (Pedersen *et al.*, 1996; Meijerink y Van Wijngaarden, 1997) se ha puesto de manifiesto la presencia de comunidades microbianas bien estructuradas a diferentes profundidades, alcanzando cotas de más de 50 metros en sedimentos húmedos, de más de 2000 metros en acuíferos arenosos (Pedersen, 1993) y de aproximadamente unos 1800 metros en acuíferos graníticos (Pedersen, 1997; Brown y Goulder, 1999). Las comunidades microbianas de las capas húmedas de la tierra pueden desarrollarse tanto en las zonas no saturadas más someras como en las zonas saturadas más profundas (Madsen y Ghiorse, 1993; Pedersen, 2000; Chapelle, 2001) (Figura 1.1). Del mismo modo, estas comunidades microbianas aparecen en diversos tipos de materiales que almacenan agua bajo la superficie, como en acuíferos propiamente dichos, en acuitardos (formaciones geológicas que contienen agua en cantidades apreciables, aunque ésta circula a través de ellas con dificultad) o en acuicluidos (rocas o sedimentos en los que, aun conteniendo agua, la captación de ésta resulta económicamente inviable debido a la baja permeabilidad del material) (McMahon y Chapelle, 1991). Las comunidades microbianas de estas diferentes formaciones que acumulan agua pueden estar ecológicamente conectadas entre sí mediante el intercambio de productos de metabolismo (McMahon y Chapelle, 1991). De hecho, se han propuesto modelos de funcionamiento en sistemas acuíferos formados por diferentes materiales en los que la producción primaria, la producción secundaria y la descomposición se cierran en ciclos de no más de tres o cuatro tipos diferentes de procariotas (Pedersen, 1997; Pedersen, 2000). En cualquier caso, las evidencias actuales indican que la mayor parte de los tipos fisiológicos de microorganismos que se conocen y que se han descrito en los sistemas superficiales pueden aparecer también en los sistemas subsuperficiales (Ghiorse y Wilson, 1988; Griebler y Lueders, 2009).

Las comunidades microbianas de los acuíferos están formadas por individuos que pueden permanecer flotando en el agua intersticial de la roca saturada (microorganismos planctónicos) o que pueden estar anclados a los granos de los materiales que forman la roca cargada de agua (microorganismos bentónicos) (Madsen y Ghiorse, 1993; Gounot, 1996) (Figura 1.1). Diversos estudios han puesto de manifiesto las diferentes proporciones entre microorganismos planctónicos y bentónicos, así como las diferentes funciones que parecen llevar a cabo en el metabolismo general

del sistema acuífero en el que se desarrollan (Lehman *et al.*, 2001). Actualmente, parece confirmarse que los microorganismos bentónicos presentan mayor densidad, biomasa y actividad que los planctónicos, si bien todo ello puede depender de diversas condiciones geológicas y fisicoquímicas del sistema acuífero (Alfreider *et al.*, 1997). Una mayor biomasa y una mayor actividad de los microorganismos bentónicos puede deberse fundamentalmente a la formación por su parte de biopelículas (*biofilms*) (Sinsabaugh *et al.*, 1991; Lehman y O'Connell, 2002), las cuales actúan como una especie de trampa de nutrientes y de red de reciclado de los mismos, lo que supone una gran ventaja en ambiente oligotróficos, como suelen ser los acuíferos siempre que no hayan sufrido procesos de contaminación, tanto difusa como puntual. Sin embargo, otros trabajos demuestran que las diferencias estructurales y funcionales observadas entre ambos tipos de microorganismos pueden simplemente reflejar los diferentes papeles ecológicos que desempeñan en la descomposición de materia orgánica y de compuestos xenobióticos en los acuíferos (Lehman y O'Connell, 2002), sin que ninguno de ellos tenga que ser más relevante que el otro. En este sentido, estudios recientes demuestran que existe un perfecto equilibrio entre las poblaciones de microorganismos planctónicos y bentónicos en los sistemas acuíferos (Goldscheider *et al.*, 2006).

La importancia a nivel funcional de las comunidades microbianas de los acuíferos viene determinada principalmente por las actividades metabólicas de los procariotas, que se relacionan directamente con los ciclos biogeoquímicos de la materia. En el ciclo del carbono, los estudios sobre descomposición de materia orgánica, producción bacteriana de carbono y actividades enzimáticas extracelulares, han concluido que los microorganismos conforman uno de los principales compartimentos de este ciclo en los sistemas acuíferos y en los sedimentos húmedos más someros (Chróst, 1989; Chróst y Rai, 1993; Miettinen *et al.*, 1996; Álvarez y Guerrero, 2000; Álvarez, 2002; Kolehmainen *et al.*, 2009); además, con respecto al ciclo del carbono, se tiene constancia también de actividades metanogénicas y metanotrofas (Sundh *et al.*, 1994; Raskin *et al.*, 1996; Dojka *et al.*, 1998; Kleikemper *et al.*, 2002; López-Archilla *et al.*, 2007). En el ciclo biogeoquímico del nitrógeno, diversos estudios han puesto de manifiesto la importancia de los microorganismos de las aguas subterráneas en los procesos de nitrificación y desnitrificación en acuíferos (Bengtsson, 1989; Correll *et al.*, 1997; Hu *et al.*, 2000; Santoro *et al.*, 2006; Smith *et al.*, 2006). En el ciclo biogeoquímico del fósforo, los estudios con comunidades microbianas acuáticas subsuperficiales se han desarrollado sobre todo en sistemas marinos (Hoppe, 2003), poniéndose de manifiesto igualmente la importancia central de éstas en las transformaciones que se llevan a cabo en este ciclo. Es decir, la diversidad metabólica que presentan las comunidades microbianas en los acuíferos es muy elevada, siendo muchas veces comparable a la que presentan en los sistemas naturales superficiales tanto terrestres como acuáticos (Madsen y Ghiorse, 1993; Griebler y Lueders, 2009).

Los primeros estudios sobre la diversidad de las comunidades microbianas en las aguas subterráneas se basaron en técnicas de cultivo (Ghiorse y Wilson, 1988; Madsen y Ghiorse, 1993; Balkwill *et al.*, 1997). Este tipo de técnicas reveló una diversidad muy limitada de procariotas altamente relacionados con heterotrofos superficiales muy bien conocidos, entre los que destacaron miembros de *Proteobacteria*, *Bacteroidetes*, *Actinobacteria* y *Firmicutes* (Boivin-Janhs *et al.*,

1995; Griebler y Lueders, 2009). Estos primeros estudios concluyeron que los procariotas con ciclos de vida más sencillos eran más abundantes y estaban más ampliamente distribuidos en capas más profundas de los sistemas acuíferos, mientras que los microorganismos filamentosos y los formadores de esporas o de cistos estaban más restringidos a los primeros horizontes del suelo (Madsen y Ghiorse, 1993). Con el desarrollo de las técnicas independientes de cultivo aplicadas al análisis de la diversidad de las comunidades microbianas, ésta pudo ser estudiada de una manera mucho más real en las aguas subterráneas (Griebler y Lueders, 2009). Gracias a estas técnicas, se ha podido comprobar, por ejemplo, la existencia de filotipos que viven en sistemas subterráneos con más frecuencia que en sistemas superficiales (Boivin-Janhs *et al*., 1996; Pedersen *et al*., 1996; Dojka *et al*., 1998). Actualmente, los mayores esfuerzos en los estudios sobre diversidad de procariotas en los sistemas acuíferos se están dirigiendo a conocer los papeles ecológicos de ésta en las aguas subterráneas, especialmente en los ciclos biogeoquímicos y en los procesos de resistencia y resiliencia de los acuíferos frente a perturbaciones de origen antrópico (Griebler y Lueders, 2009).

En términos generales, la mayor parte de los acuíferos son heterotróficos desde un punto de vista del metabolismo del sistema; en este sentido, el mantenimiento de sus comunidades microbianas depende principalmente de los aportes de materia orgánica que se filtra desde los sistemas superficiales, tanto terrestres como acuáticos, con los que se relacionan (Hancock *et al.*, 2005; Griebler y Lueders, 2009). La cantidad, y sobre todo la calidad, de la materia orgánica en los sistemas acuíferos podrán variar en función de la velocidad de flujo del agua así como de su tiempo de permanencia en el sistema, condicionando, junto con la diversidad de aceptores de electrones, las tasas metabólicas. En caso de una entrada masiva de materia orgánica al sistema acuífero, las condiciones anóxicas podrían prevalecer sobre las óxicas (Wilson *et al.*, 1983), aunque dependiendo también del tipo de mecanismos energéticos presentes.

Por tanto, actualmente se sabe que los microorganismos colonizan los sistemas acuíferos, que las comunidades microbianas de estos sistemas subsuperficiales consisten fundamentalmente en bacterias y, en menor medida, arqueas, aunque también aparecen protozoos y hongos, y que estas comunidades están activas y juegan un papel central en los ciclos biogeoquímicos (Griebler y Lueders, 2009). De hecho, comparando la biomasa de procariotas entre diferentes hábitats de la biosfera, Whitman *et al*. (1998) proponen que entre un 6 y un 40% del total de biomasa de procariotas en el planeta podría estar en los sistemas subsuperficiales. Las características concretas de los sistemas acuíferos determinan la presencia de unas u otras comunidades microbianas y controlan la abundancia y la actividad de los microorganismos. El tamaño de grano, la profundidad o la superficie total para poder anclarse suelen considerarse como las variables que en mayor medida determinan las distribuciones espaciales y temporales de las comunidades microbianas en los sistemas subsuperficiales (Ghiorse y Wilson, 1988; Madsen y Ghiorse, 1993). Otro tipo de variables como el pH, la temperatura, la presencia de agua utilizable y de nutrientes, la disponibilidad tanto de aceptores como de donadores de electrones, la presión hidrostática y la presencia de gradientes redox influyen igualmente en esas distribuciones (Ghiorse y Wilson, 1988; Madsen y Ghiorse, 1993).

El reconocimiento de los sistemas acuíferos como ecosistemas y el de sus comunidades microbianas como miembros de complejas biocenosis representa por tanto un cambio de paradigma que se ha producido a lo largo de 75 años y, sobre todo, a lo largo de los últimos 30 (Goldscheider *et al*., 2006). Incluso, la Directiva Marco del Agua de la Comisión Europea dice que "…*el estado ecológico de una masa de agua subterránea puede determinar la calidad ecológica de una masa de agua superficial o de un ecosistema terrestre*…" (EC, 2000). En España, sin embargo, los acuíferos siguen siendo considerados, y consecuentemente estudiados, como simples reservorios subterráneos de agua. Los primeros trabajos desarrollados en España que abordaron el estudio de comunidades microbianas en las aguas subterráneas, tuvieron como objetivo lograr medidas de gestión para evitar y prevenir, en la medida de lo posible, la formación de costras biológicas, compuestas principalmente por bacterias, en las infraestructuras destinadas a la captación de aguas subterráneas para consumo humano (Senderos, 2001). El enfoque que poseían estos trabajos fue entonces muy diferente del que persigue esta tesis doctoral. Por tanto, el presente trabajo puede considerarse totalmente pionero en España.

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CAPÍTULO 3. La estructura de las comunidades microbianas del sistema acuífero de Doñana I: una aproximación intensiva

3. ENVIRONMENTAL FACTORS CONTROLLING THE SPATIOTEMPORAL DISTRIBUTION OF MICROBIAL COMMUNITIES IN A COASTAL, SANDY AQUIFER SYSTEM (DOÑANA, SOUTHWEST SPAIN)

Hydrogeology Journal (2009), 17, 767-780

ABSTRACT

Currently, aquifers are considered to be ecosystems interchanging materials and energy with other systems located in their surroundings. The aquifer system of Doñana (southwest Spain) has been studied over recent decades from a hydrogeological point of view, although nothing is known about its biological or ecological aspects. In order to describe the general characteristics of its microbial communities, bacterial abundance, cell biomass, bacterial biomass and microbial activities of functional groups were investigated by sampling over a two-year period 13 wells located in the vicinity of four very productive shallow lakes in the most superficial part of this coastal, sandy aquifer system. Multivariate analysis of variance (MANOVA) indicated differences in abundance and biomass variables among piezometers, seasons and piezometers × seasons. Principal component analysis showed that temperature and dissolved oxygen appeared to be the most important factors controlling the temporal variability of microbial communities. Hydrological connectivity between surface water and groundwater was important in the control of the spatiotemporal distribution of microbial communities. Due to this hydrological connection, the aquifer system and the wetlands constitute a unique entity, a unique ecosystem, called *hydroecosystem*, where microbial communities could play a central ecological role.

3. FACTORES AMBIENTALES QUE CONTROLAN LA DISTRIBUCIÓN ESPACIOTEMPORAL DE LAS COMUNIDADES MICROBIANAS EN UN SISTEMA ACUÍFERO SEDIMENTARIO Y COSTERO (DOÑANA, SUROESTE DE ESPAÑA)

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RESUMEN

Actualmente, los acuíferos se consideran ecosistemas que intercambian materia y energía con otros sistemas localizados en sus proximidades y con los que se relacionan. El sistema acuífero de Doñana (suroeste de España) ha sido profundamente estudiado durante los últimos años desde un punto de vista hidrogeológico, si bien no se conoce nada acerca de sus características biológicas y ecológicas. Con el fin de describir por primera vez las características de sus comunidades microbianas, se han estudiado en las aguas subterráneas más someras de este sistema acuífero arenoso y costero la abundancia bacteriana, la biomasa celular, la biomasa bacteriana y la actividad microbiana de algunos grupos funcionales durante un período de dos años y en trece piezómetros localizados en las orillas de cuatro lagunas muy productivas. El análisis multivariante de la varianza (MANOVA) ha constatado la existencia de diferencias significativas en la abundancia y en la biomasa entre piezómetros, estaciones y piezómetros × estaciones. El análisis de componentes principales ha puesto de manifiesto que tanto la temperatura como el oxígeno disuelto parecen ser dos de las principales variables que controlan el patrón temporal de distribución de las comunidades microbianas planctónicas del acuífero. La conexión hidrológica entre las aguas subterráneas y las aguas superficiales parece tener un control fundamental en la distribución espaciotemporal de las comunidades microbianas. Debido a esta conexión hidrológica, el sistema acuífero y los humedales constituyen una única entidad, un único ecosistema, llamado *hidroecosistema*, en el que las comunidades microbianas juegan un papel ecológico central.

INTRODUCTION

Nowadays, aquifer systems are considered to be ecosystems full of life with their microbial communities, mainly bacterial communities, playing essential roles in geobiological processes (McMahon, 2001; Danielopol *et al*., 2003). Since the 1960s, the relationships between abiotic factors and microbial communities in aquifer systems have been studied and a new line of research, called the *ecology of groundwaters*, has been developed (Hancock *et al*., 2005). The paradigm in the study of aquifer systems changed from a pure hydrogeological view to a more ecological perspective, considering these systems as open systems, mainly heterotrophic, and permanently interchanging materials and energy with other ecological systems located in the vicinity (Danielopol, 1989; Baker *et al*., 2000). The ecological approximation to the study of aquifer systems can be applied both to shallow and deep aquifer systems, although each type of ecosystem can display different ecological features. In any case, aquifer systems function following general ecological principles and display similar complex ecological processes to those occurring in surface aquatic systems (Danielopol, 1994).

Although the first studies about microbial ecology in aquifer systems focused on descriptive aspects of microbial communities, the ecological perspective was rapidly introduced in subsequently published articles (Franklin *et al*., 1999). As a result, some effects of microbial communities on the geochemistry of aquifer systems have been described (McMahon and Chapelle, 1991; Murphy *et al*., 1992; Bennett *et al*., 2000; Roling *et al*., 2001; McMahon, 2001; Schryver *et al*., 2006; Mauck and Roberts, 2007) and several factors controlling the distribution, diversity and activity of microbial communities have also been characterized (Murphy *et al*., 1992; Phelps *et al*., 1994; Zhang *et al*., 1997; Franklin *et al*., 1999; Griebler *et al*., 2002; Zhou *et al*., 2004; Ball and Crawford, 2006). However, long-term studies about the spatiotemporal distribution of microbial communities in shallow aquifer systems are scarce.

The present study was carried out in the aquifer system of Doñana (southwest Spain). This aquifer has been studied from a hydrogeological point of view (Trick and Custodio, 2004). Hydrological connectivity between groundwaters and surface waters is also well studied in Doñana (Sacks *et al*., 1992). Several studies exist about the ecology of the surface aquatic systems (Serrano and Toja, 1995). Moreover, some studies performed in these surface aquatic systems indicate the great importance of microorganisms in their general functioning (López-Archilla *et al*., 2004). However, there is a total absence of studies exploring the biological and ecological aspects of the aquifer system of Doñana. The microbial communities of this aquifer system could have an important role in the general functioning of the greater fluvial-littoral ecosystem of Doñana (please, see site description) where different terrestrial and aquatic ecosystems depend on groundwater (Manzano *et al*., 2007). In order to understand the possible ecological roles of microbial communities in a more general context it is necessary to identify interactions occurring within and among the surrounding environments over a range of scales (Bennett *et al*., 2000; Hancock *et al*., 2005). This study should be considered as a first step towards a detailed, ecological description of microbial communities inhabiting the aquifer system of Doñana.

Consequently, the main purposes of this paper are: (1) to describe microbial communities in the upper part of the aquifer of Doñana, in terms of bacterial abundance and bacterial biomass over a two-year period, in the surroundings of four very productive shallow lakes, (2) to explain the possible factors that control the spatial and temporal distribution of these microbial communities, (3) to show the main active functional groups of microorganisms present in these communities, and (4) to present the aquifer microbial communities in a more general ecological framework as defined by the different ecosystems within Doñana.

MATERIALS AND METHODS

Site description

Doñana National and Natural Parks, declared Biosphere Reserves, Ramsar Sites and Natural World Heritage Sites, are located on the southwest coast of the Iberian peninsula (Figure 3.1). They are included in the greater fluvial-littoral ecosystem of Doñana (2200 km^2), a wide system of marshes, dunes and beaches associated with the coastal geomorphologic dynamic of the Guadalquivir river mouth. The greater fluvial-littoral ecosystem of Doñana encompasses four different types of ecosystem: marshes, aeolian mantles, coastal lines and the Guadalquivir river estuary (Montes *et al*., 1998). The climate of the area is predominantly mediterranean sub-humid, with dry summers and wet winters. Average annual rainfall is around 525 mm, but the variability between and within years is notable; most precipitation occurs in autumn and spring.

There are more than 600 different shallow lakes located over the aeolian mantles in Doñana (Coleto, 2003). The ecological importance of these shallow lakes has been highlighted several times (Serrano and Toja, 1995; Álvarez, 2002; Coleto, 2003). Previous studies, carried out during 1998 and 1999 in Santa Olalla, Dulce, Toro and Oro shallow lakes by staff of the Universidad Autónoma de Madrid, measured physical, chemical and microbiological variables both in the water column and in the shallow sediments. Spatiotemporal distributions for all variables were observed. In the water column, mean temperatures ranged between 18.9 and 22.3 ºC, mean dissolved oxygen values ranged between 1.53 and 9.51 mg L^{-1}, mean pH data ranged between 6.71 and 9.51, mean ammonium concentrations ranged between 0.07 and 0.99 mg L^{-1}, mean nitrate data ranged between 0.00 and 0.08 mg L^{-1}, mean soluble reactive phosphorus values ranged between 0.00 and 0.02 mg L^{-1}, and mean total phosphorus concentrations ranged between 0.08 and 0.64 mg L^{-1} (Álvarez, 2002; Coleto, 2003). Primary production, carried out entirely by phytoplankton, reached peaks close to 3000 mg C m^{-3} h^{-1} (López-Archilla *et al*., 2004). Finally, mean bacterial abundances estimated in the sediments of these shallow lakes ranged between 4.47×10^8 bact mL^{-1} and 8.87×10^9 bact mL^{-1}, while mean cell biomasses ranged between 63 and 106 femtograms of carbon (fgC) (Álvarez, 2002).

The aquifer system of Doñana extends over an area close to 3000 km^2 (Figure 3.1). The aquifer system consists of unconsolidated plio-quaternary detrital sediments overlapping pliocene and miocene silts and marls that constitute the impermeable lower boundary of the system. The total thickness varies between a few meters inland to more than 200 m at the coast. In general,

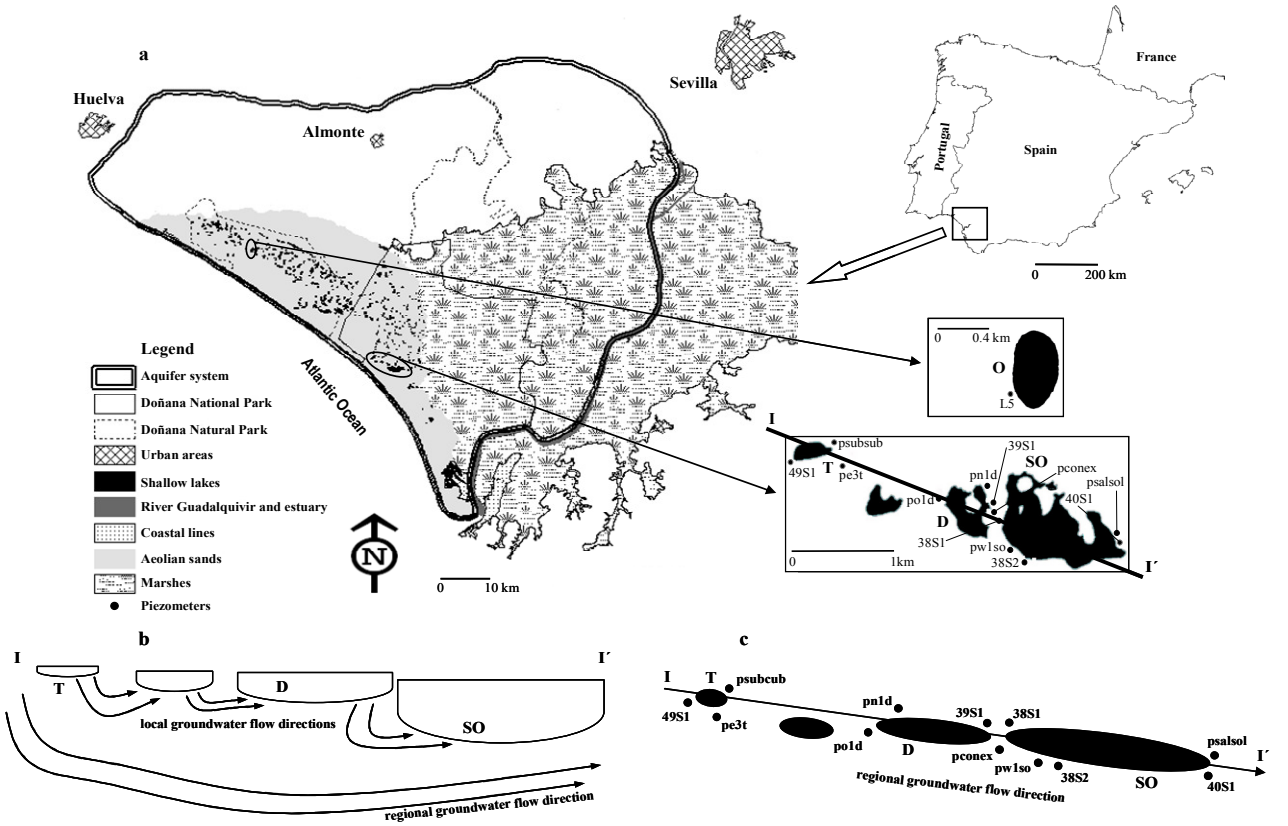


Figure 3.1 Geographical location of the greater fluvial-littoral ecosystem of Doñana showing the aquifer system limit, the Doñana National Park limit, the Doñana Natural Park limit, the limits of the four different types of ecosystems and both studied piezometers and shallow lakes (O, Oro; T, Toro; D, Dulce; SO, Santa Olalla) (a). Diagram showing both the hydrological connections between the shallow lakes and the general regional groundwater flow direction (not to scale) (see section I-I´) (b). Shallow lakes and studied piezometers in relation to the regional groundwater flow direction (c). Lake Oro does not appear in b and c because it is not located within the main regional groundwater flow direction (not to scale).

permeability values range from 1-3 m d^{-1} up to 200 m d^{-1} (Trick and Custodio, 2004). The most permeable materials (alluvial and aeolian sands with interlayered gravels) crop out to the west and north of the system. To the east and southeast, these materials become confined under some 50-70 m of thick Pleistocene estuarine and marshy clays (Manzano and Custodio, 2007). From a hydrodynamic point of view, this aquifer is confined under the low-permeability (mean 0.01 m d^{-1}) environment of clayey marsh sediments, while it is phreatic under the aeolian mantles. In the aeolian mantles, there are two different lithological domains (called upper and lower units) that allow for the presence of two different and interconnected hydrodynamic units (Trick and Custodio, 2004). The thick, fine to medium sand deposits of the upper unit form an upper, phreatic, and relatively low permeability aquifer (permeability around 2-3 m d^{-1}) that contains the water table and which overlies a lower, heterogeneous, and semiconfined aquifer, comprised of coarse sand and gravels. The hydraulic transmissivity of the lower, thinner aquifer is higher (from 70 up to 150 m^2 d^{-1}) than that of the upper aquifer (values between 0.5 and 2 m^2 d^{-1}). Between the upper and the lower units there is an intermediate layer of grey clays and fine to medium clayey sands containing iron oxide minerals (Trick and Custodio, 2004). Recharge occurs via the direct infiltration of rainfall in the phreatic areas. Natural discharge takes place to the ocean, to the rivers and ravines, to

many small phreatic shallow lakes placed on top of the aeolian mantles and through phreatic evapotranspiration (Coleto, 2003).

Table 3.1 Location and hydrogeological features of the selected piezometers

Piezometers	Altitude (mbls)[1]	Depth (mbls)[2]	Screen depth (mbls)[2]	UTM X (29)[3]	UTM Y (29)[3]	Series[4]	Location[5]	Hydrogeological behaviour[6]
38S1	5.70	17.48	14.20-17.00	724420	4095800	SGOP	SO-D	inflowing
38S2	6.00	11.20	8.10-11.00	724490	4095425	SGOP	SO-D	mainly outflowing
39S1	5.80	21.96	18.00-21.70	724107	4095773	SGOP	SO-D	inflowing
40S1	6.30	24.19	6.40-9.40	725165	4095562	SGOP	SO-D	
49S1	14.90	14.80	11.40-14.20	721412	4096836	SGOP	T	
L5	68.00	15.00	8.00-10.00	705087	4111029	L	O	
psubcub	13.20	2.50	1.80-2.30	721570	4096527	puam	T	
pe3t	14.50	3.80	2.80-3.30	721855	4096926	puam	T	
po1d	5.50	4.40	3.40-3.90	723727	4095863	puam	SO-D	inflowing
pn1d	6.00	3.00	2.00-2.25	724092	4095848	puam	SO-D	
pconex	6.30	2.70	2.20-2.40	724186	4095769	puam	SO-D	
psalsol	6.00	3.75	3.50-3.75	725178	4095599	puam	SO-D	outflowing
pw1so	6.50	2.90	2.00-2.25	724408	4095430	puam	SO-D	mainly outflowing

[1]Meters above sea level
[2]Meters below land surface
[3]Universal Transport Mercator units in meters
[4]Piezometer series: SGOP and L5 (deep and wide piezometers); puam (shallow and narrow wells)
[5]Shallow lakes: SO, Santa Olalla; D, Dulce; O, Oro; T, Toro
[6]Main hydrogeological flow direction for wells located in the vicinity of shallow lakes: *inflowing* means that water flows predominantly towards the pond, while *outflowing* is towards the aquifer

Sampling procedure: physical and chemical groundwater variables

Groundwater samples were collected once per season in the same month over two years (winter 2003-winter 2005) from 13 piezometers situated over the aeolian mantles and in the surroundings of four very productive shallow lakes (Oro, Toro, Dulce and Santa Olalla). These wells, except well L5, are located along the direction of the regional groundwater flow (Figure 3.1). The piezometer screens used for water collection were designated as shallow (< 5m, puam-series) or deep (> 5m, SGOP-series and L5) (Table 3.1). The lithology of the piezometer screens, consistent in all of them, is made up of medium to fine aeolian sands (Trick and Custodio, 2004). From a hydrogeological point of view, the studied piezometers show some differences. Some wells, located in areas where the groundwater usually feeds the shallow lakes, are called inflowing piezometers, while other wells, located in areas where water from the shallow lakes usually moves towards the groundwater, are called outflowing piezometers. However, the hydrogeological behaviour of each well depends on the amount of precipitation in the area during the hydrological year, and usually changes between different seasons (Sacks *et al*., 1992).

Samples were collected following standard procedures for both chemical (Dunlap *et al*., 1977) and microbiological variables (Fredrickson and Phelps, 1997). To avoid mixing processes between groundwater and non-flowing piezometer water, a pneumatic pump was employed (Danielopol and Niederreiter, 1987). Groundwater was pumped from each borehole until the temperature, dissolved oxygen, pH and electric conductivity readings stabilized. All physical parameters were measured with a WTW 340i handheld multi-parameter device.

Ammonium, nitrate, nitrite, soluble reactive phosphorus and total phosphorus were estimated by standard methods (APHA *et al*., 1987). Alkalinity was estimated by standard methods (APHA *et*

al., 1987) during the same sampling day. Ferrous and ferric iron concentrations, as well as total iron, were determined by the ferrozine colorimetric method (Viollier *et al*., 2000).

Microbiological variables

Groundwater samples for microbiological variables were collected from each well in triplicate, stored in 100 mL polyurethane bottles (pre-washed in 5% HCl and distilled water) and fixed with formaldehyde (2% v/v final concentration); these samples were kept in the dark at 4 ºC until the filtration process was begun. Bacterial abundance was determined by epifluorescence microscopy after staining with DAPI (4',6-diamidino-2-phenylindole-dihydrochloride) 1 μg mL^{-1} final concentration (Fry, 1990). Three different filters were prepared for each piezometer per season. Twenty different fields were viewed and counted for each filter with an Olympus IX50 inverted microscope (Kirchman *et al*., 1982). The results of the counts for bacterial abundance are presented as bact mL^{-1} groundwater. Cell biovolume was obtained by measuring the lengths and widths of bacterial cells under a magnification of ×1000 in the same filters as where the bacterial abundance was determined. Sixty cells were measured in each filter and were classified as cocci, small and large rods and filamentous bacteria (Kirchman *et al*., 1982). The biovolumes of cells were calculated assuming that the shapes of the cells were either spheres or cylinders with hemispheres on both sides (Fry, 1990). Linear dimensions were converted to volumetric units using geometric formulas. Cell biomass was calculated from the cell biovolume using a conversion factor based in the allometric model with the formula $C = 120V^{0.7}$, where C = cell biomass in fgC and V = cell biovolume in μm^3 (Psenner, 1993). The product of the bacterial abundance and the cell biomass is the bacterial or population biomass, which is shown as μgC mL^{-1} groundwater.

Biological activity reaction tests (BART™), manufactured by Droycon Bioconcepts Inc. (Regina, SK, Canada), were used to detect iron-related (IRB), sulphate-reducing (SRB), denitrifying (DNB) and nitrifying (NB) microbial, mainly bacterial, activities (Cullimore, 1993). Samples for analysing the activity of microbial functional groups were stored in autoclaved 100 mL glass bottles. Analyses began during the same sampling day. Three 10 mL-replicates of groundwater were incubated during 10 days at room temperature for each of the functional groups. The first day after the incubation in which the activity was detected, was selected as the beginning of the microbial activity. Assays were run during winters and summers (2003, 2004).

Other variables

Rainfall data were obtained with permission (Spanish Meteorological Service, AEMET) from a sampling weather station located in Doñana National Park. Piezometric levels were measured prior to sampling with a water level acoustic indicator. Air temperature data were obtained, with permission, from a weather station located in the Doñana Biological Reserve. The water temperature of the shallow lakes was seasonally measured with a WTW 340i handheld multi-parameter device.

Table 3.2 Summary of physical and chemical variables measured during the study period. All variables show three rows per piezometer: first row displays total number of seasonal samples (n) (bearing in mind lost samples or measures, respectively for chemical analyses and physical parameters), second row exhibits mean and standard deviation, and third row shows the range (T, temperature; DO, dissolved oxygen; EC, electric conductivity)

Piezometers	T (ºC)	DO (mg L^{-1})	pH	EC (µS cm^{-1})	Ammonium (mg L^{-1} $N\text{-}NH_4^+$)	Nitrate (mg L^{-1} $N\text{-}NO_3^-$)
38S1	24	24	24	24	25	22
	18.90 (1.36)	2.26 (1.11)	6.70 (0.27)	225.08 (28.69)	1.24 (1.39)	0.35 (0.28)
	16.30-22.30	0.80-3.80	6.21-7.20	180.00-278.00	0.03-4.43	0.04-1.01
38S2	24	24	24	24	19	16
	19.13 (1.48)	1.62 (1.08)	6.87 (0.46)	719.89 (387.14)	0.37 (0.38)	0.21 (0.14)
	17.10-22.40	0.48-4.94	5.52-7.50	340.00-1209.00	0.01-1.14	0.09-0.54
39S1	24	24	24	24	22	25
	19.09 (1.31)	2.46 (1.69)	6.70 (0.36)	192.29 (25.09)	1.21 (0.87)	0.42 (0.43)
	15.20-21.30	0.57-9.00	5.97-7.52	150.00-221.00	0.02-3.37	0.00-1.32
40S1	24	24	24	24	25	22
	18.93 (1.23)	2.07 (1.18)	6.54 (0.72)	400.15 (308.06)	0.46 (0.51)	0.34 (0.49)
	17.20-22.00	0.70-4.20	4.40-7.11	179.00-1134.00	0.01-1.66	0.10-2.47
49S1	24	24	24	24	25	22
	18.40 (1.20)	1.68 (1.05)	5.62 (0.43)	872.89 (336.40)	3.05 (2.40)	0.61 (0.57)
	13.80-19.40	0.70-5.71	5.00-6.50	437.00-1377.00	0.54-8.21	0.16-2.69
L5	24	24	24	24	25	22
	19.32 (1.24)	1.64 (0.68)	5.84 (0.22)	377.96 (177.06)	1.86 (2.53)	0.14 (0.15)
	16.70-22.10	0.64-3.43	5.13-6.10	214.00-700.00	0.08-8.30	0.00-0.58
psubcub	24	24	24	24	22	25
	19.25 (3.88)	3.49 (0.87)	6.24 (0.32)	546.63 (335.45)	0.04 (0.04)	1.20 (0.62)
	12.70-24.90	2.20-4.82	5.72-6.81	186.00-1319.00	0.00-0.14	0.26-2.12
pe3t	24	24	24	24	19	25
	18.50 (2.68)	6.44 (1.04)	6.53 (0.76)	232.46 (44.49)	0.06 (0.08)	1.50 (1.02)
	14.70-21.90	5.29-8.90	5.40-8.03	176.00-331.00	0.01-0.27	0.38-4.94
po1d	24	24	24	24	25	25
	18.60 (2.71)	1.23 (0.77)	6.98 (0.23)	278.63 (28.64)	0.94 (0.23)	0.18 (0.15)
	13.80-22.40	0.32-3.30	6.49-7.37	225.00-316.00	0.06-1.22	0.06-0.79
pn1d	24	24	24	24	19	25
	18.65 (2.57)	2.84 (0.60)	6.55 (0.26)	185.43 (37.52)	0.04 (0.03)	1.64 (1.10)
	14.40-21.20	2.02-4.40	6.16-7.25	129.00-240.50	0.00-0.14	0.66-6.11
pconex	24	24	24	24	25	25
	19.97 (3.12)	1.72 (0.79)	6.28 (0.72)	220.63 (90.25)	0.79 (0.99)	0.94 (0.57)
	16.00-23.70	0.32-3.08	3.27-7.23	149.00-441.00	0.00-3.83	0.24-3.37
psalsol	24	24	24	24	25	22
	19.39 (2.31)	1.06 (0.58)	6.44 (1.05)	4068.96 (2454.18)	0.35 (0.11)	0.13 (0.05)
	13.50-15.80	0.19-2.34	3.65-7.09	616.00-6600.00	0.18-0.55	0.02-0.20
pw1so	24	24	24	24	19	16
	20.68 (4.71)	1.56 (0.65)	6.68 (0.12)	775.15 (301.88)	4.47 (1.40)	0.25 (0.11)
	13.20-27.50	0.68-3.72	6.53-6.90	432.00-1173.00	3.03-7.59	0.08-0.41

Table 3.3 Summary of chemical variables measured during the study period. All variables show three rows per piezometer: first row displays total number of seasonal samples (n) (bearing in mind lost samples or measures, respectively for chemical analyses and physical parameters), second row exhibits mean and standard deviation, and third row shows the range (SRP, soluble reactive phosphorus; TP, total phosphorus; Fe, total iron)

Piezometers	SRP (mg L^{-1} $P\text{-}PO_3^{4-}$)	TP (mg L^{-1} P)	Fe (mg L^{-1} Fe)	Ferrous iron (mg L^{-1} Fe^{2+})	Ferric iron (mg L^{-1} Fe^{3+})	Alkalinity (meq L^{-1})
38S1	25	25	23	14	23	23
	0.30 (0.35)	0.37 (0.37)	0.90 (0.61)	0.14 (0.23)	0.81 (0.55)	0.93 (0.21)
	0.03-1.03	0.00-1.17	0.09-2.64	0.00-0.78	0.09-2.62	0.60-1.20
38S2	19	19	17	14	17	17
	0.05 (0.05)	0.16 (0.07)	4.61 (2.93)	1.73 (1.66)	3.39 (3.45)	2.50 (0.97)
	0.00-0.13	0.01-0.29	1.50-12.05	0.03-5.10	0.55-12.05	0.90-4.00
39S1	25	25	23	14	23	23
	0.17 (0.16)	0.25 (0.29)	1.33 (2.03)	0.14 (0.32)	1.25 (2.02)	0.85 (0.31)
	0.02-0.70	0.01-1.02	0.07-9.82	0.00-1.23	0.13-9.82	0.20-1.38
40S1	25	25	23	20	23	23
	0.10 (0.08)	0.14 (0.08)	0.99 (0.87)	0.28 (0.47)	0.88 (0.80)	0.86 (0.27)

Table 3.3 Continued

Piezometers	SRP (mg L^{-1} P-PO_3^{4-})	TP (mg L^{-1} P)	Fe (mg L^{-1} Fe)	Ferrous iron (mg L^{-1} Fe^{2+})	Ferric iron (mg L^{-1} Fe^{3+})	Alkalinity (meq L^{-1})
	0.01-0.25	0.00-0.27	0.16-3.86	0.00-1.34	0.12-3.72	0.51-1.24
49S1	25	23	23	20	23	23
	0.01 (0.01)	0.03 (0.02)	5.53 (5.71)	1.07 (1.23)	4.60 (5.27)	0.45 (0.13)
	0.00-0.04	0.00-0.09	0.22-21.95	0.00-3.45	0.10-20.87	0.20-0.71
L5	23	25	23	17	23	23
	0.07 (0.08)	0.19 (0.17)	3.17 (1.80)	1.52 (1.50)	2.07 (1.92)	0.83 (0.42)
	0.00-0.30	0.00-0.56	0.61-7.91	0.01-3.97	0.02-7.90	0.20-1.40
psubcub	25	25	23	14	23	23
	0.02 (0.01)	0.07 (0.03)	1.22 (1.07)	0.13 (0.31)	1.14 (1.06)	0.47 (0.20)
	0.01-0.04	0.01-0.15	0.19-3.93	0.00-1.21	0.16-3.93	0.20-0.86
pe3t	25	25	23	17	23	21
	0.01 (0.00)	0.03 (0.02)	1.11 (1.47)	0.10 (0.25)	1.04 (1.42)	0.48 (0.19)
	0.00-0.01	0.00-0.06	0.11-5.88	0.00-1.07	0.11-5.76	0.20-0.71
po1d	25	25	23	23	23	23
	0.01 (0.01)	0.05 (0.03)	25.46 (11.26)	18.40 (13.57)	7.06 (6.15)	1.58 (0.43)
	0.00-0.03	0.01-0.12	11.31-64.24	1.21-63.30	0.28-24.21	0.51-2.13
pn1d	25	25	23	17	23	23
	0.01 (0.00)	0.04 (0.02)	0.44 (0.40)	0.05 (0.07)	0.40 (0.39)	0.53 (0.14)
	0.00-0.02	0.01-0.08	0.05-1.79	0.00-0.23	0.05-1.79	0.20-0.74
pconex	25	25	23	14	23	23
	0.03 (0.02)	0.09 (0.08)	2.29 (1.30)	0.72 (0.94)	1.89 (1.11)	0.64 (0.26)
	0.00-0.06	0.01-0.37	0.34-5.83	0.00-2.96	0.34-4.04	0.20-1.12
psalsol	25	25	23	23	23	23
	0.04 (0.00)	0.14 (0.07)	9.35 (3.42)	4.95 (3.73)	4.42 (3.37)	4.84 (1.24)
	0.00-0.11	0.02-0.37	3.42-15.41	0.39-12.37	0.58-10.87	3.00-7.00
pw1so	19	19	17	17	15	17
	0.17 (0.16)	0.37 (0.14)	43.89 (25.94)	41.08 (27.08)	3.33 (4.00)	3.68 (0.50)
	0.00-0.49	0.02-0.59	19.31-97.44	16.58-97.44	0.22-13.18	2.98-5.00

Statistical analysis

A MANOVA (multivariate analysis of significance) with three microbiological variables (bacterial abundance, cell biomass and bacterial biomass) and two factors (piezometer and season) was performed to search for differences in the means among piezometers, seasons and piezometers × seasons. Univariate F-tests were also carried out after MANOVA testing. Tukey´s honestly significant difference test (HSD test) was employed, as an *a posteriori* method, to compare pairs of means after MANOVA testing. The relationships among measured variables were explored using Pearson product moment correlations or Spearman rank order correlations, depending upon whether the data set in question was parametric or nonparametric in nature. Differences in mean temperature, dissolved oxygen, conductivity, pH, bacterial abundance, cell biomass, bacterial biomass, ammonium, nitrate, soluble reactive phosphorus, total phosphorus, total iron and inorganic N/P rate between 2003 and 2004 were analysed with Student t tests. Differences in microbial activities among denitrifying, iron-related and sulphate-reducing bacteria were tested for each season with a one-way ANOVA. Differences in microbial activities among seasons were explored for each functional group with a one-way ANOVA. Tukey´s honestly significant difference test (HSD test) was employed, as an *a posteriori* method, to compare pairs of means after one-way ANOVA testing. A principal component analysis (PCA), with the different seasons as the grouping variable, was carried out as an ordination method to compare different variables across seasons and to seek temporal patterns. A discriminant analysis, with the different wells as the grouping variable, was performed in order to classify and discriminate among the centroids for the different wells, and

to determine which environmental variables best accounted for differences among the wells. Data were standardized for PCA and discriminant analysis (Legendre and Legendre, 1998). Normality was examined using the Kolmogorov-Smirnov test and the variables were transformed when necessary, and when possible. Homogeneity of variances was tested using the Levene test (Zar, 1998). A p value of 0.05 was set as the significant threshold for all the statistical analyses. All statistical analyses were performed with Statistica 6.0 for Windows.

RESULTS

Physical and chemical groundwater variables

Groundwater dissolved oxygen (DO) values have shown that this aquifer system, at least in the vicinity of some productive shallow lakes, remained oxygenated during most of the seasonal sampling campaigns, although groundwater from some boreholes during some seasons displayed values close to those defining microaerophilic conditions (Table 3.2). Moreover, a seasonal pattern was observed in the DO data, with higher values during the winter or spring, just after the aquifer´s annual recharge, than during the summer or autumn. Groundwater temperature (T) was less variable throughout the year in deep piezometers than in shallow boreholes. Measured pH values were fairly consistent throughout the two years in all the wells, with values ranging between 6 and 7; however, some piezometers exhibited lower pH values, closer to 5 (Table 3.2). Most wells showed mean conductivity values (EC) between 200 and 400 μS cm^{-1}; however, a few wells displayed higher conductivity values in all seasons (Table 3.2). Among these physical groundwater variables, only mean temperature values were significantly higher during 2003 than during 2004 (t-test; $t_{0.05(2),271}$ = 2.840, p = 0.005).

Ammonium concentrations (mg L^{-1} $N\text{-}NH_4^+$) were higher than nitrate concentrations (mg L^{-1} $N\text{-}NO_3^-$) in the majority of the wells (Table 3.2). Soluble reactive phosphorus (SRP, mg L^{-1} $P\text{-}PO_4^3$) was always found and reached elevated values, close to 1 mg L^{-1} in some piezometers (Table 3.3). Total phosphorus (TP, mg L^{-1} P) values were also high in some wells (Table 3.3). Ferrous and ferric concentrations reached values close to 20 mg mL^{-1} in some seasons. Total alkalinity was measured as ~1 meq L^{-1}, except in some piezometers (Table 3.3). Mean differences between years were only significant for SRP (t-test; $t_{0.05(2),97}$ = 1.474, p = 0.002) and inorganic N/P rate (t-test; $t_{0.05(2),97}$ = 1.569, p = 0.019); in 2003 SRP levels were lower than during 2004, while inorganic N/P rates were higher than during 2004.

Microbial abundance and biomass

Microscope counts showed only prokaryotic organisms. As a consequence, they seem to dominate the upper part of this aquifer system. A clear predominance exists of short rod-shaped bacteria over cocci and filamentous bacteria. The average bacterial abundance in the studied piezometers was $1.35 \pm 1.16 \times 10^7$ bact mL^{-1} groundwater for all boreholes in all seasons. Figures 3.2a and 3.2b show the seasonal changes in cell abundance during the study period. There were significant differences among different piezometers and among seasons (MANOVA and univariate F-tests; Tables 3.4 and 3.5). A clear temporal pattern could be described for the bacterial abundance of this aquifer system

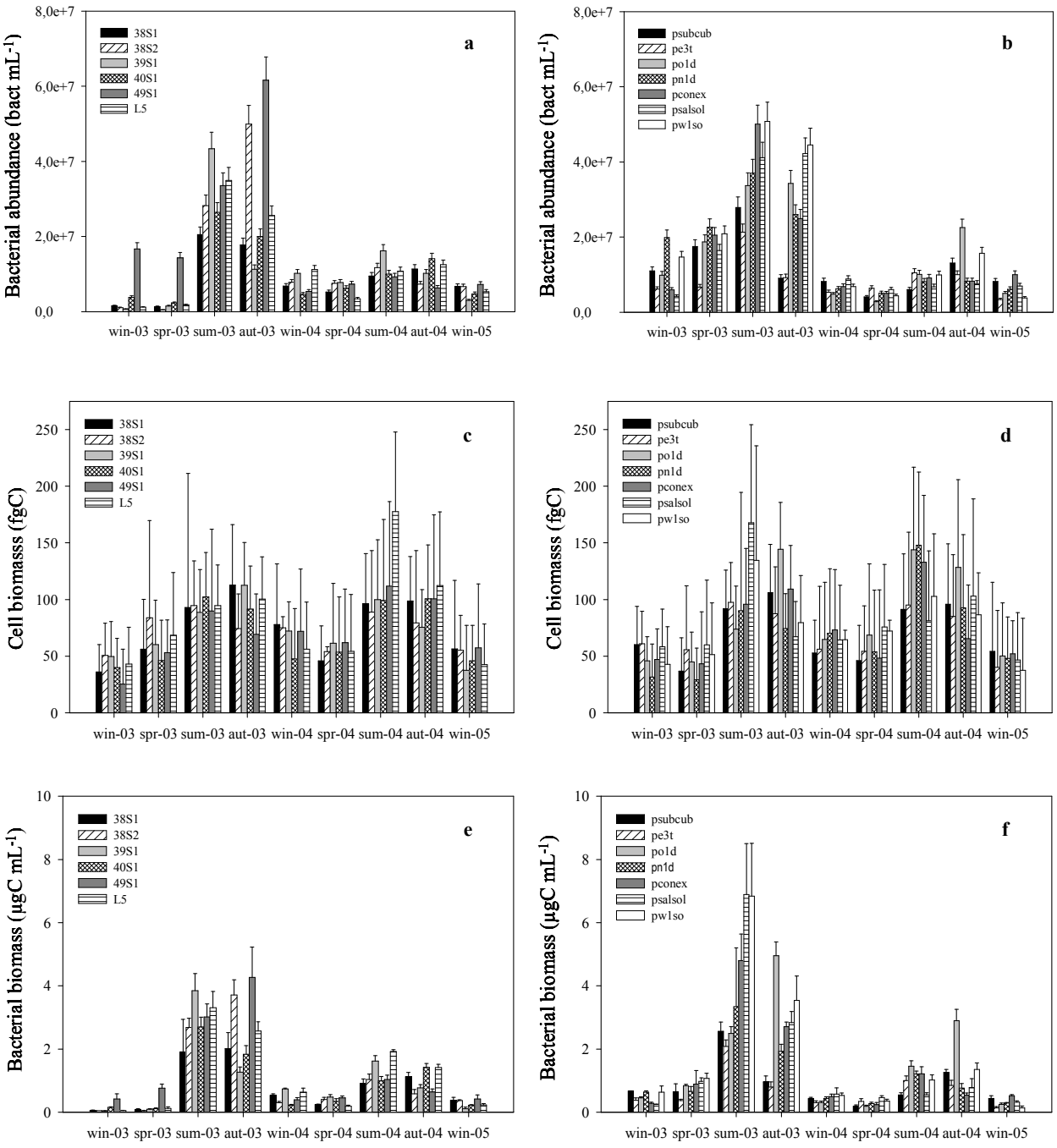


Figure 3.2 Seasonal changes in microbiological variables during the study period, both for shallow piezometers (< 5m; puam-series; right plots) and for deep wells (> 5m; SGOP-series and L5; left plots). Bacterial abundance (a and b), cell biomass (c and d), bacterial biomass (e and f) (win, winter; spr, spring; sum, summer; aut, autumn; solid bars denote standard deviation). All means (columns) and standard deviations (bars) have been calculated on triplicate samples from each piezometer.

(Figure 3.2, a and b). During the summer and the autumn of 2003 the piezometers displayed the highest values (HSD tests, $p = 0.000$), without exception, while during the winters (2003, 2004 and 2005) and the springs (2003 and 2004) the piezometers showed the lowest values, without statistical differences among the latter five seasons (HSD tests, $p \geq 0.274$). In summer and autumn 2004, the bacterial abundance of all the boreholes was significantly higher than during the winter and the spring of the same year (HSD tests, $p \leq 0.045$), but lower than the abundance found during the

summer and the autumn of 2003 (HSD tests, p = 0.000). The mean bacterial abundance was significantly higher during 2003 than during 2004 (t-test; $t_{0.05(2),414}$ = 4.213, p = 0.006). Bacterial abundance positively correlated with T (r = 0.518, p = 0.000, n = 117). Moreover, bacterial abundance in all boreholes taken together during each season correlated with the precipitation from two months prior to sampling (Spearman rank order correlation, r = -0.711, p < 0.048, n = 9). No correlation was found between bacterial abundance and depth.

Table 3.4 Results after MANOVA testing

Source of variation	Effect df	Error df	Wilk´s Lambda	F	p
Piezometers	36	1031.887	0.003	175	0.000
Seasons	24	1012.807	0.000	774	0.000
Piezometers × seasons	288	1047.713	0.000	48	0.000

Table 3.5 Univariate F-tests results after MANOVA testing

Source of variation	df	MS	F	p
Piezometers	12			
Bacterial abundance		0.490	1.90	0.029
Cell biomass		0.025	3.00	0.000
Bacterial biomass		0.561	67.97	0.000
Residual	351	0.250		
Seasons	8			
Bacterial abundance		5.010	19.80	0.000
Cell biomass		1.238	151.40	0.000
Bacterial biomass		9.832	1191.71	0.000
Residual	351	0.008		
Piezometers × seasons	96			
Bacterial abundance		0.300	12.40	0.037
Cell biomass		0.035	4.30	0.000
Bacterial biomass		0.300	36.37	0.000
Residual	351	0.009		

Mean cell biomass was determined to be 75.04 ± 29.78 fgC. Seasonal changes are shown in Figures 3.2c and 3.2d. Statistically significant differences for cell biomass values among the piezometers were found (MANOVA and univariate F-tests; Tables 3.4 and 3.5). There was a clear temporal pattern, with statistical differences detected among seasons (MANOVA and univariate F-tests; Tables 3.4 and 3.5). During 2003, an increase in cell biomass was observed from winter to autumn in the upper part of the aquifer (Figure 3.2, c and d); significantly higher values were found in summer and autumn than in winter and spring (HSD tests, p = 0.000). During 2004 a very similar temporal pattern was observed. The highest cell biomasses were observed in summer 2004 (HSD tests, p = 0.000). Moreover, values were significantly higher during the winter, spring and summer of 2004 than during the same seasons in 2003 (HSD tests, p = 0.000). Unlike bacterial abundance, mean cell biomass was significantly higher during 2004 than 2003 (t-test; $t_{0.05(2),414}$ = -3.867, p = 0.000). Cell biomass positively correlated with T (r = 0.712, p = 0.000, n = 117), ammonium (r = 0.202, p = 0.018, n = 107) and bacterial abundance (r = 0.498, p = 0.000, n = 117). No correlation was found between cell biomass and rainfall. No correlation was found between cell biomass and depth. Average bacterial biomass was 1.40 ± 0.72 μgC mL^{-1} for all piezometers in all seasons. Seasonal changes are shown in Figures 3.2e and 3.2f. There were statistical differences among

piezometers (MANOVA and univariate F-tests; Tables 3.4 and 3.5). Statistical differences among seasons were found during both years, and significant increases in bacterial biomass from winters to summers were observed (MANOVA and univariate F-tests; Tables 3.4 and 3.5). When corresponding seasons were compared across years, some differences were apparent; winter 2003 data showed significantly lower bacterial biomasses than winter 2004 (HSD test, $p = 0.000$) and both summer and autumn bacterial biomasses were significantly lower during 2004 than during 2003 (HSD tests, $p = 0.000$). Overall, like bacterial abundance, mean bacterial biomass was significantly higher in 2003 than in 2004 (t-test; $t_{0.05(2),414} = 2.741$, $p = 0.000$). This variable positively correlated with T ($r = 0.680$, $p = 0.000$, $n = 117$). A significant Spearman rank order correlation was found between bacterial biomass in all boreholes taken together during each season and rainfall from two months prior to each sampling period ($r = -0.736$, $p < 0.050$, $n = 9$).

Microbial activity: functional groups

Microbial activities of some functional groups, except those for nitrifying bacteria (NB), were found in this aquifer system. Significant differences, measured as the mean of the first day after incubation in which activity was detected in all wells taken together (Table 3.6), were found in all seasons (ANOVA tests, $F \geq 9.588$, $p \leq 0.001$). During 2003 and winter 2004, IRB showed the highest activities in comparison with DNB and SRB (HSD tests, $p \leq 0.003$), whose activities were not significantly different (HSD tests, $p \geq 0.089$). In summer 2004, both IRB and SRB displayed significantly higher activities than DNB (HSD tests, $p \leq 0.046$). Seasonal differences were displayed by DNB, IRB and SRB (ANOVA tests, $F \geq 7.749$, $p \leq 0.001$). Microbial activities were statistically higher during summers than during winters in both years (HSD tests, $p \leq 0.009$). No statistical differences were found between winters or between summers in the activities of DNB and IRB (HSD tests, $p \geq 0.320$), but significantly higher microbial activities of SRB were found during summer and winter 2004 than during the same seasons in 2003 (HSD tests, $p \leq 0.026$).

Table 3.6 Summary of microbial activities for different functional groups provided by BART™ tests (DNB denitrifying bacteria; IRB, iron-related bacteria; SRB, sulphate-reducing bacteria; win, winter; sum, summer). Numbers indicate first day after incubation in which microbial activity was recorded

Piezometers	DNB				IRB				SRB			
	win-03	sum-03	win-04	sum-04	win-03	sum-03	win-04	sum-04	win-03	sum-03	win-04	sum-04
38S1	10	10	10	10	5	3	5	2	9	5	9	2
38S2	10	10	6	2	5	2	4	1	9	5	5	2
39S1	10	4	10	10	5	3	4	2	9	5	10	3
40S1	10	4	10	6	4	2	5	2	9	4	6	3
49S1	3	3	7	2	4	2	5	2	9	8	10	3
L5	5	3	10	2	4	3	5	2	9	4	8	2
psubcub	9	3	10	3	8	2	7	2	10	8	10	3
pe3t	10	8	6	4	7	2	6	2	10	7	10	4
po1d	10	8	10	10	6	3	5	4	10	4	9	4
pn1d	7	5	10	2	7	2	7	2	10	4	9	6
pconex	6	5	10	2	5	2	4	2	9	5	9	6
psalsol	7	3	10	2	7	2	6	2	9	4	3	3
pw1so	10	3	10	10	8	5	9	2	9	6	4	2

Hydrology

Different hydrologic conditions occurred over the two-year period. Year 2004 showed higher seasonal piezometric levels than year 2003 because the 2003-2004 hydrological cycle was more humid than that in 2002-2003 (Figure 3.3). Bacterial abundance was significantly higher in some

outflowing/mainly outflowing piezometers (psalsol and pw1so) than in some inflowing ones (38S1 and 39S1) during winter and spring 2003 (HSD tests, $p \leq 0.047$); during the summer and the autumn of the same year, these differences were not significant (HSD tests, $p \geq 0.125$). Significantly higher cell biomasses were detected in some inflowing piezometers than in some outflowing ones during autumn 2003 (HSD tests, $p \leq 0.021$); during winter and spring 2003, differences among these wells were not significant (HSD tests, $p \geq 0.147$). During the 2004 sampling campaign, differences were not found for these microbiological variables among the piezometers located in the surroundings of the productive shallow lakes.

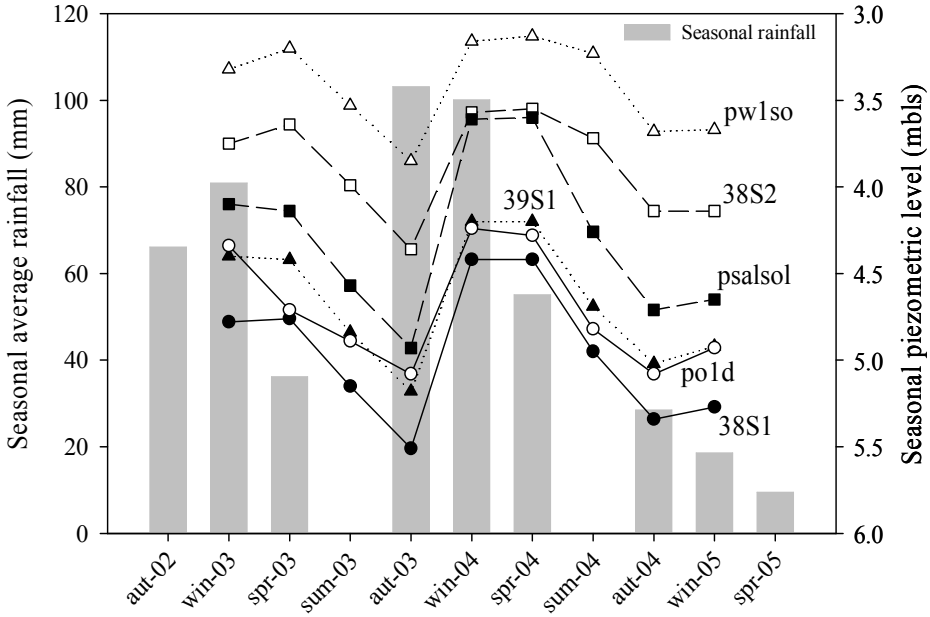


Figure 3.3 Seasonal average rainfall (mm) measured in Doñana National Park from autumn 2002 to spring 2005 (bars), and seasonal piezometric levels (in mbls, meters below land surface) measured from winter 2003 to winter 2005 in wells 38S1 and 39S1 (deep and inflowing), psalsol and pw1so (shallow and outflowing/mainly outflowing), po1d (shallow and mainly inflowing) and 38S2 (deep and mainly outflowing) (lines).

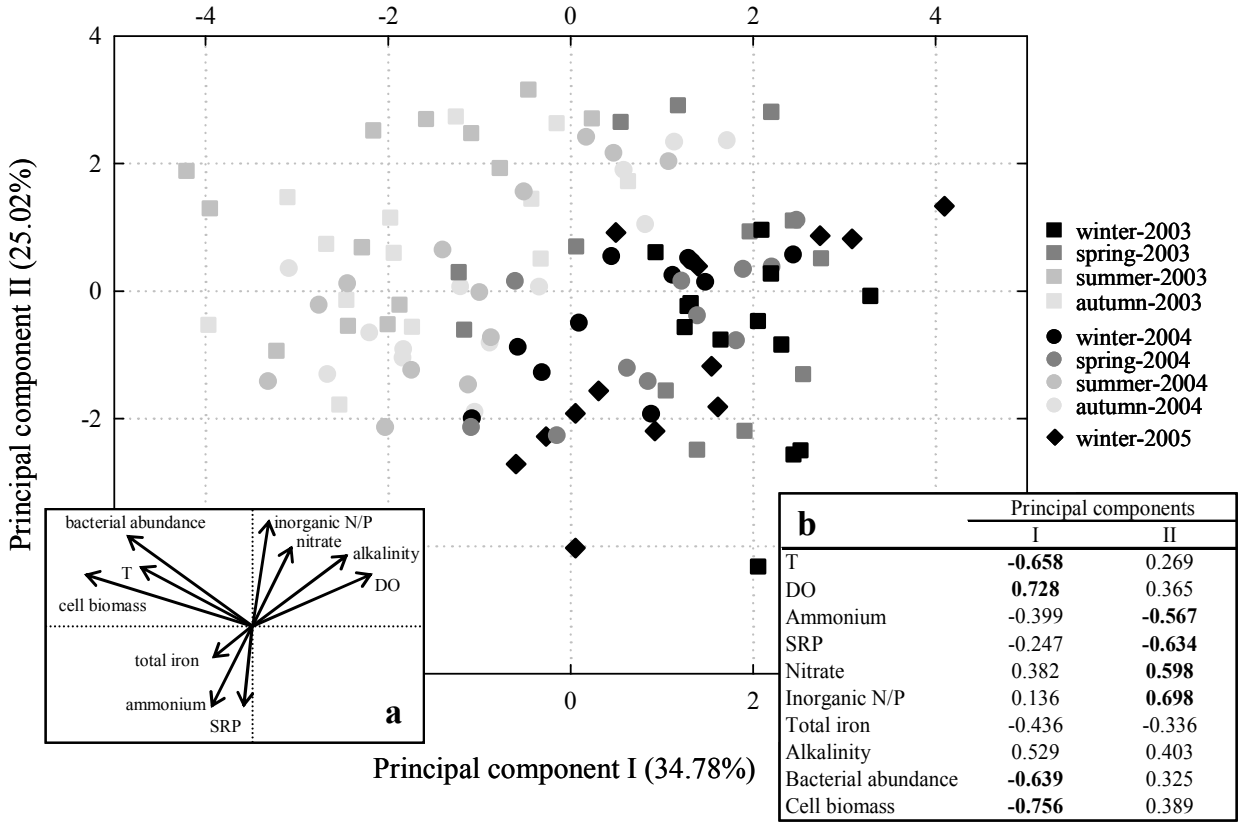
Exploratory statistical analyses

The variance explained by the first three factors included in the principal component analysis of the correlation matrix performed is 73.67% (Figure 3.4). Two microbiological variables (bacterial abundance and cell biomass) and two parameters controlling them (temperature and DO) defined the principal component I. Samples were classified into two major groups in relation to this axis I: one on the positive side (winter/spring 2003, winter/spring 2004 and winter 2005) and another on the negative side (summer/autumn 2003 and 2004). Inorganic chemical forms of nitrogen and phosphorus primarily defined the principal component II. The first three canonical axes obtained

after the discriminant analysis ($F_{108,682}$ = 7.087, p = 0.000) accounted for 78.73% of the total variance (Figure 3.5). DO, EC, nitrate, alkalinity, total iron and ammonium were the variables that maximized differences and distances for the centroids of piezometers along the first canonical function, which explained 42.34% of the total variance. Along the second canonical axis, which explains 19.60% of the total variance, microbiological parameters (bacterial abundance and cell biomass) and soluble reactive phosphorus were the variables that allowed the distribution of the centroids of the wells.

DISCUSSION

This work is the first long-term study focused on the spatiotemporal distribution of bacterial abundance, cell biomass and activity of microbial communities located in the shallower part of the coastal, sandy aquifer system of Doñana (southwest Spain). It is also, as far as the authors are aware, the first article published on this area with information about 13 wells over 9 seasons. Microbiological data provided by this study demonstrate the presence of a noteworthy microbial



b

	Principal components	
	I	II
T	**-0.658**	0.269
DO	**0.728**	0.365
Ammonium	-0.399	**-0.567**
SRP	-0.247	**-0.634**
Nitrate	0.382	**0.598**
Inorganic N/P	0.136	**0.698**
Total iron	-0.436	-0.336
Alkalinity	0.529	0.403
Bacterial abundance	**-0.639**	0.325
Cell biomass	**-0.756**	0.389

Figure 3.4 Positions of the 117 seasonal samples plotted in the reduced space of the first two principal components after principal components analysis (PCA), showing both variables projected in the plane determined by the first two principal axes (a) and factor loadings of these descriptors in both axes (b) (boldface type indicates major factor loadings in each axis) (T, temperature; DO, dissolved oxygen; SRP, soluble reactive phosphorus).

community in the upper part (saturated zone) of this coastal aquifer system. The microbial community changes its bacterial abundance and cell biomass, as well as the activity levels of some of its functional groups, throughout the year, and shows differences among locations. Bearing in

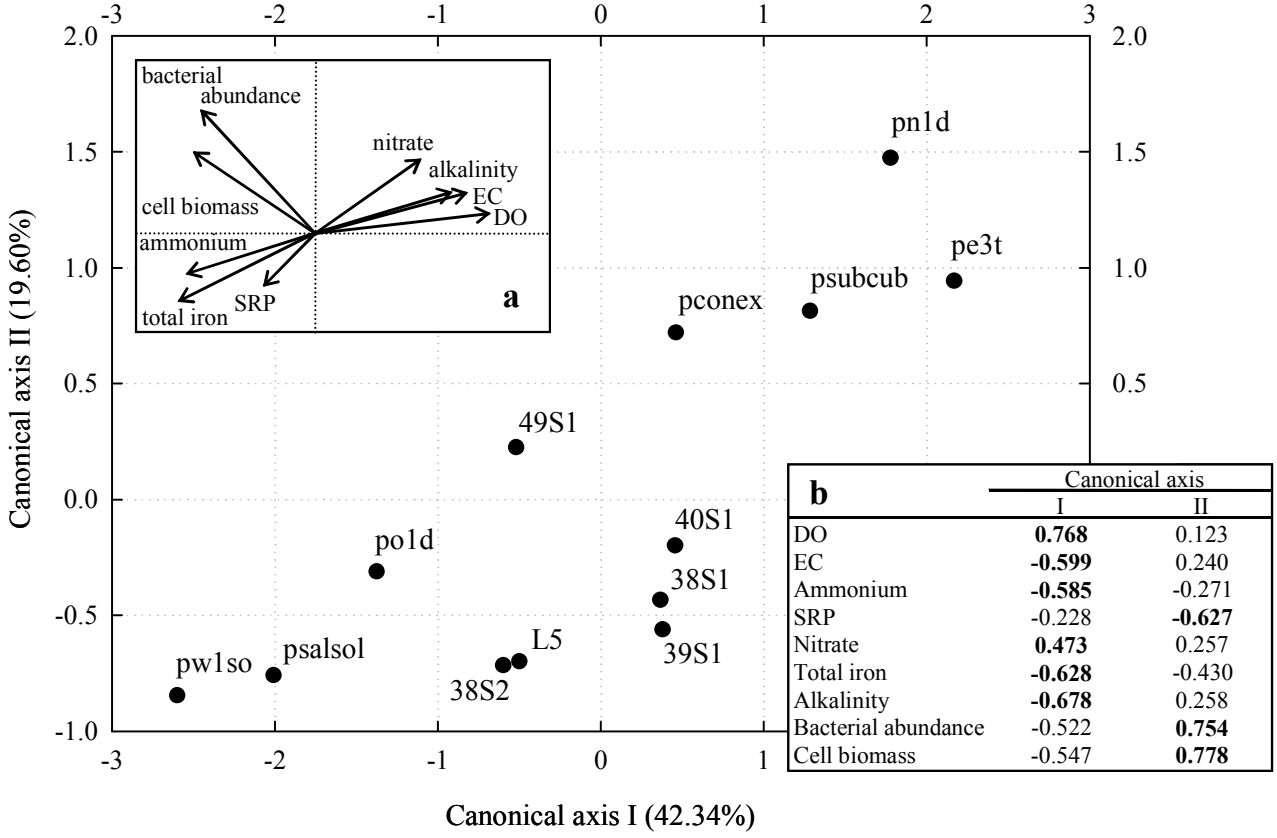
mind the absence of other microbial groups in the counted samples, prokaryotes are, by far, the most abundant microbial group in this aquifer system, as has been described elsewhere (Beloin *et al*., 1988; Bone and Balkwill, 1988; Balkwill, 1989; Balkwill *et al*., 1989; Sinclair and Ghiorse, 1989; Fredrickson *et al*., 1991; Shi *et al*., 1999).

Microbial abundance and biomass: factors controlling microbial communities in groundwater

The mean planktonic bacterial abundance of this aquifer system is higher than that observed in other similar aquifer systems, both pristine (Marxsen, 1988; Alfreider *et al*., 1997; Griebler *et al*., 2002) or contaminated (Haack and Bekins, 2000), and is more or less the same as the bacterial abundance observed for attached cells in other sandy, relatively shallow aquifer systems (Goldscheider *et al*., 2006). The high density of planktonic bacteria in Doñana´s aquifer could be explained by the good hydrological transmissivity of its upper part (Trick and Custodio, 2004), which allows a hydrological connectivity between the groundwater and surface waters (Coleto, 2003). This connectivity favours the movement of carbon and nutrients (high concentrations for N and P inorganic forms in Doñana´s groundwater were observed; Tables 3.2 and 3.3) and permits a distribution of bacteria throughout sands (Sophocleous, 2002). The average cell biomass observed for this aquifer system is in the range of values found for sandy, shallow aquifers (Griebler *et al*., 2002), although slightly higher, probably due to the seasonality of data. The mean bacterial biomass is lower for the groundwater than that observed for sediments in the shallow lakes of Doñana (Álvarez, 2002).

Although grain size and depth can explain spatial distributions of aquifer microbial communities (Musslewhite *et al*., 2003; Goldscheider *et al*., 2006; Schryver *et al*., 2006), these variables do not seem to be important in the Doñana aquifer system. There were neither significant correlations between depth and abundance nor between cell biomass and depth in any season, at least in the studied range of depths. Moreover, the lithology is very similar among wells (fine to medium aeolian sands) (Trick and Custodio, 2004) and, consequently, significant differences in microbiological variables are more difficult to find.

Hydrogeological processes have been described as important factors controlling microbial communities in aquifer systems (Fredrickson *et al*., 1995; Alfreider *et al*., 1997; Baker *et al*., 2000; Hancock *et al*., 2005). The influence of hydrogeological flows on bacterial abundances was clear in some piezometers located in the vicinity of Dulce and Santa Olalla shallow lakes. During winter and spring 2003, outflowing/mainly outflowing piezometers psalsol and pw1so showed significantly higher bacterial abundances than inflowing piezometers 38S1 and 39S1. A similar situation has been described in other sedimentary aquifer systems (Alfreider *et al*., 1997). However, during summer and autumn 2003, conditions became increasingly dry and phreatic levels dropped (Figure 3.3); in this situation, shallow outflowing/mainly outflowing piezometers (pw1so and psalsol) received less water from the shallow lakes, because this water predominantly flowed in a vertical direction towards the aquifer, to recharge it (Sacks *et al*., 1992). At the same time, deeper inflowing piezometers (38S1 and 39S1) continued receiving water with nutrients from deep aquifer areas, although probably less than in winter or spring. As a result, differences in bacterial



b

	Canonical axis I	Canonical axis II
DO	**0.768**	0.123
EC	**-0.599**	0.240
Ammonium	**-0.585**	-0.271
SRP	-0.228	**-0.627**
Nitrate	**0.473**	0.257
Total iron	**-0.628**	-0.430
Alkalinity	**-0.678**	0.258
Bacterial abundance	-0.522	**0.754**
Cell biomass	-0.547	**0.778**

Figure 3.5 Positions of the centroids of the 13 wells plotted along the first two canonical axes after discriminant analysis, showing both the contributions of the different measured variables (a) and the standardized canonical coefficients for the first two canonical variables or axes (b) (boldface type indicates main variables in each canonical axis) (DO, dissolved oxygen; EC, electric conductivity; SRP, soluble reactive phosphorus).

abundances were not significant in summer and autumn 2003. During 2004, no statistical differences were observed among piezometers probably due to a homogenization process caused by the increase in general phreatic levels after the unusually high rainfall during autumn 2003 and winter 2004 (Figure 3.3). High precipitation events have an important ecological role as a homogenization factor in the shallow lakes (Serrano and Toja, 1995). Taking into account the relevant hydrologic relation between groundwaters and surface waters in Doñana, the ecological role of high precipitation events could be applicable to groundwater as well.

The influence of hydrogeological factors on cell biomasses was less clear. During winter and spring 2003, the differences in cell biomasses between inflowing and outflowing wells were not significant, probably because the differences in quality, origin (shallow lakes or lower parts of the aquifer) and concentration of nutrients in the groundwater were not enough to distinguish differences in cell biomass between the two kinds of wells. However, in summer and autumn 2003, conditions became increasingly dry and outflowing boreholes received less water with nutrients from the shallow lakes than the inflowing wells from the lower part of the aquifer. Moreover, surface waters show less inorganic nutrient concentrations during the summer months of the year as a consequence of the observed high levels of primary production (López-Archilla *et al*., 2004), and

deep groundwaters usually show more constant levels of inorganic nutrients (Coleto, 2003). These reasons could partially explain the significant lower cell biomasses found in outflowing piezometers during autumn 2003. During 2004, spatial and temporal cell biomass patterns were unclear for comparisons between outflowing and inflowing wells. Homogenization processes in the system caused by rainfall could produce these uncertain patterns.

Spatiotemporal distribution of microbial communities

Microbial communities of the upper part of this aquifer system displayed a clear temporal pattern during both years (Figures 3.2 and 3.4). Higher temperatures seem to trigger higher bacterial abundances and cell biomasses. Similar causative relationships have been observed in other aquifer systems (Balkwill and Ghiorse, 1985; Beloin *et al*., 1988; Sinclair and Ghiorse, 1989; Bone and Balkwill, 1988; Kieft *et al*., 1998). It is important to point out that T itself could not be the only variable triggering bacterial abundances and cell biomasses in this aquifer. Positive correlations between bacterial abundance, cell biomass and bacterial biomass with T could also be explained by the increase in primary productivity rates in the shallow lakes during the summer and autumn months. Photosynthetic products could later reach the groundwater, providing the microbial communities inhabiting there with labile organic matter to fuel their metabolism (López-Archilla *et al*., 2004). Unfortunately neither primary production nor chemical variables have been measured in the shallow lakes as part of this study. Nevertheless, not all boreholes acted as outflowing wells during summer and autumn, but all of them showed increments in bacterial abundance and in cell biomass through the year (Figure 3.2). Therefore, organic matter coming from photosynthesis products does not seem to be the only reason to explain these correlations between microbiological and physical variables measured in the groundwater. Probably, a combination of variables under increasing temperatures favours higher bacterial abundances and cell biomasses. In a similar way, all functional groups detected with BART^{TM} tests showed higher activities (Table 3.6) when temperatures reached maximum values. Similar temporal patterns for bacterial activities (Haack and Bekins, 2000) have been observed in other aquifer systems.

Significant differences for bacterial abundance, cell biomass and bacterial biomass were found between years after Student t testing, as well as for temperature data. Significantly higher cell biomasses compared with lower bacterial abundances during 2004 than during 2003 could be explained by some unfavourable conditions for reproduction but not for growing during 2004. Statistically lower mean temperatures and a significantly lower inorganic N/P ratio during 2004 than during 2003, along with higher annual mean SRP and TP values during 2004 than in 2003, suggest a probable limitation by nitrogen. Several authors have discussed that under optimal conditions bacterioplankton show higher activities, grow exponentially and optimize DNA duplication with respect to protein metabolism, maximizing reproduction and showing balanced growth. In contrast, under less favourable conditions, bacterioplankton present logistic growth and protein synthesis appears to increase more rapidly than cell duplication in order to maximize survival under unfavourable environmental conditions (Petit *et al*., 1999; Pulido-Villena and Reche, 2003). In fact, under depleted nutrient conditions, elongated cells are observed (Mary *et al*., 2002).

However, other strategies observed in bacterioplankton living under nutrient limited conditions are reductive division and dwarfing (Nyström, 2004). But, these other strategies have not been observed in Doñana. The apparent contradictions between the results described in this work and the conclusions given by Nyström (2004) are due to a relative ignorance about the growth processes in bacterial cells living in nutrient-poor natural systems, because most of the knowledge about these processes comes from laboratory experiments (Mary *et al*., 2002; Nyström, 2004).

Factors working at greater spatiotemporal scales than microbial processes could also partially control the microbial abundance in this aquifer system. Mean bacterial abundance, as well as mean bacterial biomass, for all the piezometers taken together during each season showed significant, negative correlation with rainfall from two months prior to sampling, probably due to dilution processes. Inflowing piezometers 38S1 and po1d showed significant, negative correlation with rainfall from two months prior to sampling ($r = -0.435$, $p < 0.050$, $n = 9$ and $r = -0.342$, $p < 0.050$, $n = 9$, respectively), but, on the other hand, the outflowing/mainly outflowing piezometers psalsol and pw1so did not show significant correlation. Moreover, air, shallow lakes and groundwater temperatures seasonally correlated (Pearson product moment correlations, for all cases $r \geq 0.778$, $p \leq 0.01$, $n = 9$). Consequently, the influence of factors working at different scales should be kept in mind to develop a better understanding about the ecology of aquifer system microbial communities (Brockman and Murray, 1997; Musslewhite *et al*., 2003; Goldscheider *et al*., 2006). Activities in subsurface environments extend from the microscale to the macroscale, but caution in the context of interpreting field data has to be exercised (Mauck and Roberts, 2007), because it is difficult to relate bacterial processes and patterns (microscale) to climatic or hydrogeologic processes (macroscale) (Musslewhite *et al*., 2003).

The spatial distribution of microbial communities (Figure 3.5) was not as obvious as the temporal pattern. Physical and chemical variables predominantly seemed to control this distribution. This situation is not strange if it is considered that the variability of microbiological parameters in aquifer systems, even at low spatial scales, could be much larger than that for groundwater physicochemical variables at the same sample point (Brockman *et al*., 1992; Brockman and Murray, 1997; Musslewhite *et al*., 2003). Along the first canonical axis of the discriminant analysis (Figure 3.5), two different centroid groups could be distinguished relating to some chemical compounds: wells pw1so, psalsol, po1d, 38S2, L5 and 49S1 usually showed higher ammonium and total iron concentrations than pn1d, pconex, psubcub, pe3t and 40S1, and also showed significantly higher bacterial abundances during some seasons. Total iron and bacterial abundance did not significantly correlate ($r = 0.398$, $p = 0.078$, $n = 93$), but there was a significant, positive correlation between bacterial abundance and ammonium. Consequently, it is proposed that the presence of high concentrations of organic matter both in the sediments and in the water of the shallow lakes (see Coleto, 2003), as well as a rapid bacterial metabolism at the bottom of these shallow lakes (see Álvarez, 2002), could provide important amounts of ammonium and total iron as fuel for bacterial abundance and activities in the aquifer system. IRB exhibited the highest activity values during most of the seasons; as a consequence, bacteria in the upper part of the aquifer system of Doñana

could grow with compounds of organic matter as electron donors and oxidized iron as an electron acceptor. SRB also showed high levels of activity in other seasons, demonstrating that sulphate can also act as an electron acceptor. Bearing in mind that the presence of nutrients can support high bacterial abundances and activities in groundwater (Baker *et al*., 2000), the high ammonium concentrations shown by 49S1 and L5 could explain their moderate to high bacterial abundances. However, cell biomass showed a less significant correlation with ammonium and other inorganic chemical forms, suggesting that other variables could also be affecting the spatial distribution of microbial communities in the aquifer of Doñana. Among these other variables, dissolved organic carbon (DOC) or total organic carbon (TOC) could be determinants in explaining microbiological spatial patterns, as has been pointed out several times (Alfreider *et al*., 1997; Goldscheider *et al*., 2006); unfortunately, these analyses were not carried out as part of this study.

CONCLUSIONS

In summary, a notable and active microbial community, dominated by bacteria and displaying spatiotemporal changes, has been defined in the groundwater located in the surroundings of four very productive shallow lakes in the upper part of the coastal, sandy aquifer system of Doñana. Significant differences in annual mean temperature, rainfall and nitrogen and phosphorus inorganic forms provoked significant differences in groundwater microbiological variables between two years. Hydrological connectivity permits diffusion and transport for bacteria and nutrients between the sediments of the shallow lakes and the aquifer, although the microbial communities in both sites could differ because they are distinct microbial habitats (Colwell and Lehman, 1997). The high abundance of planktonic bacteria and the activity levels shown by some functional groups of bacteria illustrate the implications of this microbial community for biogeochemical cycles both in the processing of organic matter, coming mainly from the shallow lakes, and in its transport to higher trophic levels (Bennett *et al*., 2000; Hancock *et al*., 2005). Therefore, bearing in mind the hydrological connectivity between groundwater and surface water (Coleto, 2003), the control that the same physical and chemical variables seem to exert both on the microbial communities of the sediments of the shallow lakes (Álvarez, 2002) and on the groundwater, and the similarity of microbial temporal patterns observed both in the aquifer system and in the sediments of the wetlands, it is proposed that the aquifer system of Doñana is not an isolated ecological system, but is a system permanently interchanging, at different spatiotemporal scales, materials and energy with surface aquatic and terrestrial systems. Stream ecologists now accept that vertical hydrological connectivity, and not only longitudinal and lateral connectivity, controls many ecosystem processes in rivers. The hyporheic zone is now considered as part of a river ecosystem and is currently studied under holistic approximations (Sophocleous, 2002; Boulton, 2007). This work shows that, not only between rivers and hyporheic zones, but also between phreatic aquifer systems and hypogenic wetlands, hydrological connectivity is controlling some ecological processes. Surface waters, transporting bacteria, nutrients and organic matter, enter the aquifer system, where, through chemical reactions mediated by microorganisms, the groundwater chemistry can be changed before recharging surface aquatic systems again (Cullimore, 1993; Chapelle, 2001; McMahon, 2001). In

other words, microbial processes affect not only the chemistry of groundwater but also the chemistry of surface waters. As a consequence, surface waters and groundwater form a unique entity, working as a whole (Manzano and Custodio, 2007). However, wetland sciences only focus on the hydrological connections between groundwater and surface waters, while biogeochemical interactions in terms of the activities of microbial communities are not considered (Mitch and Gosselink, 2007). Therefore, it is proposed that, in order to obtain a holistic scheme, wetlands limnology should consider not only the hydrological connectivity between groundwater and surface water, but also the ecological roles of microbial communities located in aquifer systems.

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CAPÍTULO 4. La estructura de las comunidades microbianas del sistema acuífero de Doñana II: una aproximación extensiva

4. SPATIOTEMPORAL DISTRIBUTION OF MICROBIAL COMMUNITIES IN A COASTAL, SANDY AQUIFER SYSTEM (DOÑANA, SW SPAIN)

Geobiology (2009), 7, 66-81

ABSTRACT

The aquifer system of Doñana (SW Spain) represents the most important freshwater source in the Doñana Natural Area. Its spatiotemporal dynamics favour the hydrological connection between surface and subsurface ecosystems, and promote matter fluxes among the different terrestrial and aquatic systems present here. This aquifer has been intensively studied from a hydrogeological point of view but little is known from an ecological perspective. In order to understand the ecological roles played by microbial communities in this system, we conducted a long-term seasonal study of bacterial abundance, cell biomass, bacterial biomass and functional activities over a two-year period. Bacterial abundance ranged between $2.11 \pm 1.79 \times 10^5$ and $8.58 \pm 6.99 \times 10^7$ bact mL^{-1} groundwater, average cell biomass was estimated to be 77.01 ± 31.56 fgC and bacterial biomass varied between $8.99 \pm 4.10 \times 10^{-2}$ and 5.65 ± 0.70 μgC mL^{-1}. Iron-related bacteria (IRB) showed the highest activities among the functional groups studied. Moreover, among the variables that usually control spatial distributions of microbial communities in aquifer systems, depth did not have a relevant effect on this aquifer, at least in the range of depths studied, but grain size, probably due to its direct effects on hydrogeological parameters, such as permeability or porosity, appeared to exert moderate control, principally in terms of bacterial abundance. Finally, significant seasonal differences in the means of these microbiological variables were also observed; temperature seems to be the main factor controlling the temporal distribution of microbial communities in this aquifer system.

4. DISTRIBUCIÓN ESPACIOTEMPORAL DE LAS COMUNIDADES MICROBIANAS EN UN SISTEMA ACUÍFERO SEDIMENTARIO Y COSTERO (DOÑANA, SW ESPAÑA)

Geobiology (2009), 7, 66-81

RESUMEN

El sistema acuífero de Doñana (suroeste de España) constituye la fuente principal de agua dulce en el área natural de Doñana. La dinámica espaciotemporal que posee este acuífero permite la existencia de una conexión hidrológica entre las aguas superficiales y las aguas subterráneas que favorece los flujos de materia entre los diferentes sistemas acuáticos y terrestres presentes en la zona. Este acuífero ha sido intensamente estudiado desde un punto de vista hidrogeológico, aunque poco se conoce de él desde una perspectiva ecológica. Para tratar de comprender el papel ecológico que juegan las comunidades microbianas en este ecosistema, sus abundancias bacterianas, biomasas celulares, biomasas bacterianas y actividades funcionales fueron determinadas durante un periodo de dos años. La abundancia bacteriana varió entre $2.11 \pm 1.79 \times 10^5$ y $8.58 \pm 6.99 \times 10^7$ bacterias mL^{-1} agua subterránea, la biomasa celular media fue de 77.01 ± 31.56 fgC y la biomasa bacteriana osciló entre $8.99 \pm 4.10 \times 10^{-2}$ y 5.65 ± 0.70 µgC mL^{-1}. Las bacterias del hierro mostraron las mayores actividades de entre los grupos funcionales estudiados. Además, entre las variables que generalmente controlan la distribución espacial en las comunidades microbianas de los sistemas acuíferos, la profundidad no parece poseer un efecto relevante en este acuífero, al menos en el rango de profundidades estudiado, aunque el tamaño de grano, probablemente debido a sus efectos directos sobre algunas variables hidrogeológicas, tales como la permeabilidad o la porosidad, parece ejercer un moderado control, sobre todo en términos de abundancia bacteriana. Finalmente, se han encontrado algunas diferencias estacionales en las medias de las variables microbiológicas; la temperatura parece ser la variable que más controla la distribución temporal de las comunidades microbianas en este sistema acuífero.

INTRODUCTION

The paradigm in the study of aquifer systems has changed over the last few decades from a pure hydrogeological point of view to a more ecological one (Danielopol, 1989; Baker *et al.*, 2000). As a consequence, aquifer systems are currently considered to constitute a heterogeneous assemblage of discrete macro- and microscale habitats, providing a variety of living conditions (Goldscheider *et al.*, 2006).

From an ecological standpoint, aquifer systems should be regarded as open systems interchanging materials and energy with other aquatic and terrestrial systems located in the vicinity (Danielopol, 1989; Chapelle, 2001; Danielopol *et al.*, 2003). The recognition of interactions occurring within and among surrounding environments over a range of scales will allow for a better understanding of the ecological roles played by microbial communities in aquifer systems (Brockman and Murray, 1997; Bennett *et al.*, 2000; Musslewhite *et al.*, 2003; Hancock *et al.*, 2005). However, the identification of such interactions requires long-term spatiotemporal studies, very rare to date. Multidisciplinary studies would be of use in this identification, but only if all the approximations share similar spatiotemporal scales (Brockman and Murray, 1997; Musslewhite *et al.*, 2003).

Microbial ecology studies of sedimentary, relatively shallow aquifer systems are moderately abundant (Marxsen, 1988; Alfreider *et al.*, 1997; Martino *et al.*, 1998; Griebler *et al.*, 2002; Velasco Ayuso *et al*., 2008), but very few exist with large spatial and temporal scales. Some of these studies have shown aquifers as more stable ecosystems, with less spatiotemporal variation in their ecological processes, than other aquatic systems. The main goal of the present study, conducted in an area approximately of 100 km^2 over a two-year period, is to enhance our knowledge of the microbial communities existing in the aquifer system of Doñana, an ecosystem in which there have been only a few studies based upon a biological or ecological approach. This aquifer is coming to be considered as part of a great ecosystem, called the *hydroecosystem*, made up of several aquatic and terrestrial systems, and located in the Doñana Natural Area (Montes *et al.*, 1998; Manzano and Custodio, 2007; Manzano *et al.*, 2007). All these ecosystems in the Doñana Natural Area make it unique in many senses: it is a major stepping-stone in the migration route of birds moving between Europe and Africa, it is home to the most endangered mammal in the world, as well as many endemic, threatened or ecologically interesting species, and it contains what is possibly Europe's most significant wetland. Consequently, there has been an increasing number of interdisciplinary studies in the last few years, and groups of limnologists, microbiologists, hydrogeologists, economists and sociologists are working together in order to obtain a general overview of the ecological functioning of Doñana's *hydroecosystem* (Manzano *et al.*, 2007).

Bearing in mind that the most important freshwater source in the Doñana Natural Area is the groundwater, and that the aquifer controls the hydrological regime of all other ecosystems located in this area, it is important to understand the role played by microbial communities present in the aquifer system. To this end, the first step is to define these microbial communities in terms of bacterial abundance and cell biomass (Murphy and Schramke, 1998), because this information will

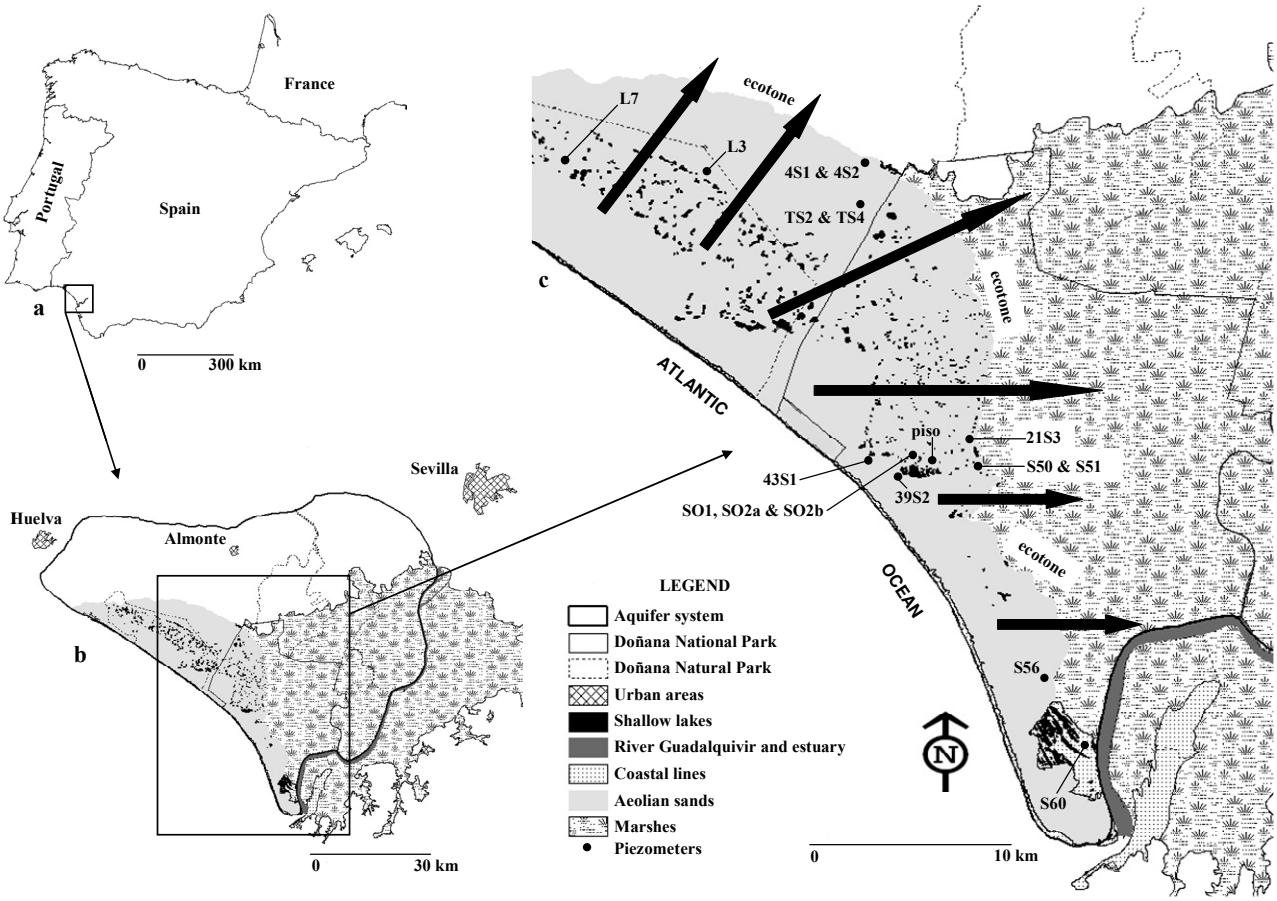


Figure 4.1 Geographical location of the Doñana Natural Area, on the southwest coast of the Iberian peninsula (a). The figure also shows the boundary of the aquifer system of Doñana, the boundaries of the Doñana National and Natural Parks, as well as the most important ecosystems in the Doñana Natural Area (b). Both shallow lakes and the 17 studied piezometers are also shown. Arrows indicate the general direction of the regional groundwater flow from east (aeolian sands) to west (marshes) (c).

enable the potential activity of the population to be identified (Bratbak, 1993). Knowledge of factors, such as depth, grain size or temperature, controlling spatial and temporal patterns in bacterial abundances, cell biomasses and microbial activities are also important to characterize the distribution and dynamics of these communities. The results of this paper complement and consolidate others resulting from a parallel, intensive study, in which microbial communities present in the groundwater located in the surroundings of four very productive shallow lakes were studied by sampling 13 piezometers, different from those employed in this paper (Velasco Ayuso *et al*., 2008).

MATERIALS AND METHODS

Site description

The Doñana aquifer system is included in the Doñana Natural Area and located in the SW atlantic coast of Spain. The aquifer has a surface area of around 3000 km^2 and hosts the Guadalquivir river marshes and Doñana National and Natural Parks, two protected natural areas of international relevance comprising approximately 1100 km^2 (Figure 4.1). They were declared Biosphere Reserves in 1981, RAMSAR Sites in 1982 and Natural World Heritage Sites in 1994. The climate

is mediterranean sub-humid with atlantic influence: dry summers and wet winters. Mean rainfall, mainly concentrated from October to March, is 500-600 mm, but has a high interannual variability. Mean yearly temperature is around 17 ºC near the coast and 18 ºC in the centre of the Natural Area (Manzano *et al*., 2007).

Table 4.1 Geographical, lithological and hydrogeological features of the selected piezometers

Piezometers	UTM X (29)	UTM Y (29)	Altitude (masl)[1]	Screen depth (mbls)[2]	Hydrogeological unit[3]	Screen lithology	Transmissivity (m^2 d^{-1})[4]	Permeability (m d^{-1})[4]	Problems[4]
piso	724598	4095905	5.50	2.00-2.25	U	fine to medium sands			
S51	727730	4097026	2.00	3.00-7.00	U	fine sands	1.13	0.28	
21S3	727969	4101313	4.00	5.40-8.20	U	fine sands			
4S1	722485	4111933	4.38	8.00-10.00	U	fine to medium sands			nitrate
L3	711081	4111414	43.00	8.00-10.00	U	fine to medium sands	0.20	0.10	
L7	700031	4113890	69.00	8.00-10.00	U	coarse sands			nitrate
39S2	723848	4095698	5.70	8.50-11.40	U	fine to medium sands			
TS4	719101	4112389	16.30	10.00-11.00	U	fine to medium sands	3.00	3.00	nitrate
43S1	722031	4096458	11.30	11.50-14.30	U	fine to medium sands	0.13	0.05	
S60	734318	4080575	3.00	16.00-17.00	U	fine sands			
TS2	719101	4112389	16.30	18.00-19.00	INT	clays	0.50	0.50	nitrate
SO2a	724189	4096032	6.00	44.00-46.00	U	fine to medium sands			
4S2	722485	4111933	4.57	36.50-43.50	L	clays and fine sands			nitrate
SO2b	724189	4096032	6.00	44.00-46.00	U	fine to medium sands			
S50	727730	4097026	3.00	52.00-60.00	L	gravels and coarse sands			
S56	733010	4087500	2.00	74.00-80.00	L	gravels and coarse sands			
SO1	724188	4096038	6.00	67.00-72.00	U	fine to medium sands			

[1]Meters above sea level
[2]Meters below land surface
[3]Location of the wells in the hydrogeological units (U, upper; L, lower; INT, intermediate)
[4]Data from Trick (1998)

The aquifer system consists of detrital, unconsolidated plio-quaternary sediments overlapping impervious miocene marine marls. The pliocene materials are impermeable marls, silts and sandy silts. The quaternary materials consist on deltaic and alluvial silts, sands and gravels to the north, and on littoral, alluvial and aeolian sands to the west. They mainly comprise amorphous silica grains. Carbonates may be present either as detrital grains or as shell remains, except in the upper part of the aeolian sand layers of the western sector (Manzano *et al*., 2007). The quaternary layers thicken from N to S and from W to SE. To the SE, the coarse sediments are covered by a thick (50-80 m) sequence of estuarine and marshy clays. The aquifer system varies in thickness, ranging from 20 m inland to over 150 m at the coast line. At regional scale, the aquifer system presents two lithologic domains: a sandy one to the N and W of the marshes, which extensive areas of aeolian sands, which roughly behaves as an unconfined aquifer, and a clayey one in the marsh area, under which a large confined aquifer is found (Manzano *et al*., 2007). In the aeolian sands, there are two different lithological sub-domains (called upper and lower units) that allow for the presence of two different hydrodynamic units. The thick and fine to medium sand deposits of the upper unit conform a relatively homogeneous phreatic aquifer that contains the water table and overlies a lower, less homogeneous, and semiconfined aquifer composed of coarse sands and gravels (Trick and Custodio, 2004). The hydraulic transmissivity of the lower, thinner aquifer is higher than that of the upper one. Between the upper and the lower units there is an intermediate layer of grey clays and fine to medium clayed sands containing iron oxide-minerals. Recharge occurs via direct rain infiltration in the aeolian sands (Trick and Custodio, 2004). Groundwater mainly flows eastward from aeolian sands in the west to the ecotone and the marsh in the east (Manzano *et al*., 2007) (Figure 4.1). Natural discharge takes place to the ocean, rivers and ravines, to many small phreatic shallow lakes situated above the aeolian mantles (Coleto, 2003), and through phreatic

evapotranspiration (Manzano and Custodio, 2007). In general, there are vertical, descending flows in recharge areas and vertical, ascending flows in discharge areas and groundwater extraction points (Trick and Custodio, 2004).

Sampling procedure: physical and chemical variables

Groundwater samples were collected seasonally (winter 2003 - winter 2005) from 17 piezometers located in an area encompassing approximately 100 km^2 (Figure 4.1). Except well piso (installed and monitored by the Universidad Autónoma de Madrid, UAM), all boreholes were installed and are monitored by the Spanish Geological Survey (IGME) and the Guadalquivir river Basin Authority (CHG). Screen depth of piezometers ranges from 3.00 to 80.00 meters below land surface (mbls) and all are located in the aeolian sands (both in the upper and in the lower unit) (Table 4.1).

Groundwater samples were collected following chemical (Dunlap *et al.*, 1977) and microbiological (Fredrickson and Phelps, 1997) standard procedures. A special pneumatic pump was employed to avoid mixing processes between groundwater and retained water in the piezometer (Danielopol and Niederreiter, 1987). Groundwater was pumped from each well until temperature, dissolved oxygen, pH and electric conductivity readings stabilized (Manzano *et al.*, 2007). All physical parameters were measured with a WTW 340i handheld multi-parameter device. Chemical parameters (alkalinity, ammonium, nitrate, nitrite, soluble reactive phosphorus and total phosphorus) were estimated by means of standard methods during the ten following days (APHA *et al.*, 1987); alkalinity was analyzed during the same sampling day (APHA *et al.*, 1987). Ferrous and ferric iron concentrations, as well as total iron, were determined by the ferrozine colorimetric method (Viollier *et al.*, 2000).

Microbiological variables

Groundwater samples for microbiological variables were collected from each well in triplicate, stored in 100 mL polyurethane bottles (pre-washed in 5% HCl and distilled water), and fixed with formaldehyde (2% v/v final concentration). Samples were stored in the dark at 4 ºC until the filtration process. Bacterial abundance was determined by epifluorescence microscopy after staining with DAPI (4',6-diamidino-2-phenylindole-dihydrochloride) 1 μg mL^{-1} final concentration (Fry, 1990). The solution was then filtered through a 0.2 μm pore size GTBP 25 mm Ø Millipore filter. Filters were transferred to a labelled microscope slide and kept frozen until samples could be counted (Bölter *et al.*, 2002). Three different filters were prepared for each sampled well per season, and twenty different fields were viewed and counted for each filter (Kirchman *et al.*, 1982) with an Olympus IX50 inverted microscope. The results of the counts for bacterial abundance are presented as bact mL^{-1} groundwater. Length and width measurements of bacterial cells under a magnification of ×1000 were used to determine the cell biovolume; sixty cells were measured in each filter and were classified as cocci, small and large rods, and filamentous bacteria. Biovolumes of bacterial cells were calculated assuming that the shapes of the cells were either perfect spheres or cylinders with hemispheres on both sides (Fry, 1990). Linear dimensions were converted to volumetric units using geometric formulas (Bölter *et al.*, 2002). Cell biomass was calculated from the cell biovolume

using a conversion factor based on the allometric model with the formula $C = 120 \times V^{0.7}$, where C = cell biomass in fgC and V = cell biovolume in μm^3 (Psenner, 1993). Cell biomass is presented as fgC. The product of the bacterial abundance and the cell biomass is the bacterial or population biomass, which is shown as $\mu gC\ mL^{-1}$ groundwater.

Evidence of microbial activity for some functional groups of bacteria (nitrifying, NB; denitrifying, DNB; iron-related, IRB; sulphate-reducing bacteria, SRB) was provided by commercial biological activity reaction tests (BART™) (Cullimore, 1993). A BART™ test is a simple and effective method for detecting the presence and the relative potential activity of a specific functional group of microorganisms. Briefly, three 10 mL-replicates of groundwater were incubated during 10 days at room temperature for each functional group. The first day following incubation in which the activity was detected, was selected as the start of activity by a specific functional group. The more time required for the detection of a specific activity, the less activity the functional group showed. Assays were carried out during winters and summers (years 2003 and 2004).

Hydrogeological variables

Transmissivity and permeability values for some piezometers were previously measured (Trick and Custodio, 2004). Rainfall data were obtained with permission (Spanish Meteorological Service, INM) from a sampling weather station located in Doñana National Park.

Statistical analyses

Differences in the means of bacterial abundance, cell biomass and bacterial biomass among wells were tested, for each sampling campaign, using a one-way MANOVA test. Temporal differences in the means of the same microbiological variables for each well sampled more than three times during the study period were also tested by means of a one-way MANOVA test. Two different MANOVAs had to be performed to test the effect of one out of the two different factors (wells or seasons) because the data matrix was not regular. In other words, not all the piezometers were sampled during the same sampling campaigns, or at least the same three sampling campaigns. However, all piezometers were taken into account for statistical purposes when spatial differences were searching. Univariate F-tests were also performed after MANOVA testing. Tukey´s honestly significant difference test (HSD test) was employed, as an *a posteriori* method, to compare pairs of means after MANOVA testing. Differences in the means for microbial activities of functional groups were tested, for each sampling campaign, using a one-way ANOVA test. Temporal differences in the means of microbial activities during both years for each functional group were tested using a one-way ANOVA test. Tukey´s honestly significant difference test (HSD test) was employed, as an *a posteriori* method, to compare pairs of means after ANOVA testing. Homogeneity of variances was tested with the Levene test (Zar, 1998). Relationships among measured variables were explored using Pearson product moment correlations or Spearman rank order correlations, depending upon whether the dataset in question was parametric or nonparametric in nature. Normality was examined by using the Kolmogorov-Smirnov test, and variables were

transformed when necessary, and when possible. Relationships between physicochemical (temperature, dissolved oxygen, pH, electric conductivity, ammonium, nitrate, soluble reactive phosphorus, total phosphorus, ferric iron, ferrous iron, total iron and alkalinity) and microbiological (bacterial abundance, cell biomass and bacterial biomass) variables were explored throughout a canonical correlation analysis; Bartlett´s χ^2 test was used for testing the significance of the correlations between any pairs of the canonical variates (Quinn and Keough, 2002). A principal component analysis (PCA), with seasons as the grouping variable, was performed as a multivariate ordination method to compare different seasons and to detect temporal patterns. Data were standardized for PCA (Legendre and Legendre, 1998). A p value of 0.05 was set as the significant threshold for statistical analyses. All statistical analyses were performed with Statistica 6.0 for Windows.

RESULTS

Physicochemical variables

Groundwater temperature (T) showed mean values of approximately 19 ºC; these values increased throughout the year during both years (Table 4.2). Dissolved oxygen (DO) exhibited mean values of 2 mg L^{-1}, although with a different temporal pattern, with higher winter and spring values than those for summer or autumn. Therefore, T and DO negatively correlated ($r = -0.341$, $p = 0.004$, $n = 71$). The pH was very constant and ranged from 6 to 7 in most of the wells (Table 4.2). Electric conductivity values (EC) were measured around 300-400 µS cm^{-1}; however, in some piezometers located in the ecotone, close to the marsh (Figure 4.1), conductivities were higher.

Table 4.2 Physical variables and alkalinity seasonally measured in the different wells (mean ± SD) (T, temperature; DO, dissolved oxygen; EC, electric conductivity; win, winter; spr, spring; sum, summer; aut, autumn)

Piezometers	Seasons	T (ºC)	DO (mg L^{-1})	pH	EC (µS cm^{-1})	Alkalinity (meq L^{-1})
piso	sum-03	19.90 ± 0.26	2.30 ± 0.10	6.76 ± 0.24	202.20 ± 0.26	0.61 ± 0.12
	aut-03	20.63 ± 0.35	1.83 ± 0.06	7.16 ± 0.15	172.52 ± 1.41	0.50 ± 0.01
	sum-04	22.53 ± 0.15	2.57 ± 0.05	6.39 ± 0.01	263.67 ± 2.08	0.57 ± 0.06
	aut-04	21.30 ± 0.10	2.16 ± 0.00	6.51 ± 0.02	210.67 ± 3.21	0.53 ± 0.06
	win-05	15.90 ± 0.20	1.97 ± 0.01	6.78 ± 0.07	235.67 ± 7.37	0.27 ± 0.11
S51	win-03	14.90	5.48	8.30	366.00	
	spr-03	19.70 ± 0.28	1.90 ± 0.49	7.89 ± 0.01	303.67 ± 0.09	1.92 ± 0.01
	aut-03	21.07 ± 0.41	1.07 ± 0.04	8.14 ± 0.11	276.00 ± 2.64	1.95 ± 0.06
	spr-04	18.03 ± 0.15	1.97 ± 0.07	8.15 ± 0.13	246.37 ± 0.57	1.43 ± 0.08
	aut-04	20.17 ± 0.05	1.26 ± 0.00	8.21 ± 0.01	339.67 ± 17.95	1.60 ± 0.00
	win-05	18.70 ± 0.10	1.86 ± 0.02	8.04 ± 0.00	303.67 ± 3.06	1.27 ± 0.11
21S3	win-03	17.40	1.12	7.33	184.10	
	spr-03	18.65 ± 0.63	1.49 ± 0.48	7.15 ± 0.21	177.95 ± 7.70	0.87 ± 0.01
	sum-03	22.23 ± 0.05	1.40 ± 0.17	7.67 ± 0.01	169.27 ± 1.25	1.22 ± 0.10
	aut-03	21.97 ± 0.40	0.97 ± 0.15	7.50 ± 0.12	168.30 ± 0.98	0.97 ± 0.08
4S1	spr-03	19.30 ± 0.04	1.75 ± 0.07	7.30 ± 0.16	290.50 ± 0.26	0.45 ± 0.00
	sum-03	20.07 ± 0.05	0.77 ± 0.15	7.12 ± 0.14	326.70 ± 8.46	2.31 ± 0.28
	aut-03	20.03 ± 0.40	0.47 ± 0.12	7.65 ± 0.41	313.00 ± 6.92	2.05 ± 0.05
	aut-04	18.17 ± 0.15	0.92 ± 0.02	7.83 ± 0.00	250.00 ± 1.02	2.08 ± 0.08
	sum-04	20.50 ± 0.19	1.26 ± 0.04	7.08 ± 0.01	300.00 ± 2.64	1.40 ± 0.20
	aut-04	18.83 ± 0.37	2.57 ± 0.00	7.31 ± 0.00	299.33 ± 1.15	1.60 ± 0.00
	win-05	18.30 ± 0.40	2.59 ± 0.01	7.09 ± 0.01	264.00 ± 0.00	1.07 ± 0.23

Table 4.2 Continued

Piezometers	Seasons	T (ºC)	DO (mg L^{-1})	pH	EC (µS cm^{-1})	Alkalinity (meq L^{-1})
L3	sum-03	19.45 ± 0.07	3.55 ± 0.04	6.08 ± 0.07	74.42 ± 3.79	0.40 ± 0.00
	spr-04	19.33 ± 0.05	4.36 ± 0.07	5.87 ± 0.01	66.33 ± 1.52	0.55 ± 0.02
L7	win-03	17.30	6.20	6.03	796.00	
	sum-03	19.36 ± 0.20	2.40 ± 0.09	6.54 ± 0.07	760.50 ± 6.50	1.46 ± 0.24
	sum-04	22.10 ± 0.09	1.49 ± 0.00	6.90 ± 0.03	463.33 ± 1.52	1.27 ± 0.12
	aut-04	19.43 ± 0.11	1.56 ± 0.00	6.51 ± 0.00	474.33 ± 0.57	1.30 ± 0.10
39S2	win-04	17.33 ± 0.15	2.74 ± 0.10	6.62 ± 0.01	136.67 ± 4.72	0.72 ± 0.17
	spr-04	19.27 ± 0.05	4.89 ± 0.04	6.78 ± 0.02	125.67 ± 0.57	0.52 ± 0.5
	sum-04	20.27 ± 0.15	1.86 ± 0.07	6.44 ± 0.00	134.67 ± 0.57	0.60 ± 0.00
	aut-04	20.53 ± 0.15	3.29 ± 0.00	6.64 ± 0.01	169.33 ± 1.52	0.47 ± 0.06
	win-05	17.80 ± 0.20	2.89 ± 0.01	6.70 ± 0.02	166.67 ± 0.58	0.40 ± 0.00
TS4	win-03	18.80	5.75	6.19	228.00	
	sum-03	20.63 ± 0.75	4.50 ± 0.17	5.67 ± 0.06	225.00 ± 3.35	0.55 ± 0.01
	aut-03	19.50 ± 0.09	2.70 ± 0.05	6.02 ± 0.07	214.67 ± 8.38	0.54 ± 0.05
	win-04	18.27 ± 0.05	1.57 ± 0.05	7.64 ± 0.06	233.33 ± 3.05	0.61 ± 0.06
43S1	win-03	17.30	3.54	6.70	950.00	
	spr-03	20.55 ± 0.35	3.08 ± 0.05	6.29 ± 0.01	374.66 ± 1.06	0.53 ± 0.00
	sum-03	19.57 ± 0.05	0.67 ± 0.48	6.16 ± 0.05	232.67 ± 1.15	1.42 ± 0.13
	aut-03	19.23 ± 0.15	3.00 ± 0.34	7.09 ± 0.21	147.67 ± 10.96	0.53 ± 0.04
S60	win-03	18.20	2.68	7.82	1668.00	
	win-04	18.33 ± 0.15	1.63 ± 0.05	7.55 ± 0.06	7020.00 ± 30.69	19.97 ± 0.06
	win-05	19.60 ± 0.00	2.19 ± 0.01	7.84 ± 0.01	16400.00 ± 10.00	10.00 ± 0.00
TS2	win-03	18.80	4.73	5.88	278.00	
	sum-03	20.77 ± 0.25	2.77 ± 0.15	5.78 ± 0.01	275.00 ± 0.61	0.64 ± 0.04
	aut-03	19.27 ± 0.05	1.46 ± 0.13	6.35 ± 0.14	279.00 ± 1.25	0.44 ± 0.12
	win-04	17.03 ± 0.32	3.93 ± 0.15	5.94 ± 0.06	170.33 ± 0.70	1.91 ± 0.08
SO2a	spr-03	18.70 ± 0.14	2.05 ± 0.07	6.62 ± 0.03	148.67 ± 0.05	0.53 ± 0.00
	aut-03	21.40 ± 0.14	1.71 ± 0.00	7.02 ± 0.01	188.33 ± 1.52	1.05 ± 0.05
	spr-04	17.77 ± 0.15	2.51 ± 0.02	6.92 ± 0.01	161.33 ± 1.98	0.99 ± 0.08
	sum-04	19.70 ± 0.09	2.11 ± 0.01	6.71 ± 0.01	244.67 ± 8.02	0.67 ± 0.12
	aut-04	19.17 ± 0.20	1.93 ± 0.05	6.88 ± 0.00	202.00 ± 2.56	0.53 ± 0.12
	win-05	18.60 ± 0.10	2.48 ± 0.05	6.68 ± 0.01	198.67 ± 0.58	0.53 ± 0.11
4S2	win-03	18.10	3.36	7.69	328.00	
SO2b	spr-03	18.75 ± 0.35	1.50 ± 0.28	6.51 ± 0.07	183.00 ± 0.26	0.52 ± 0.00
	aut-03	20.60 ± 0.01	1.11 ± 0.00	6.90 ± 0.03	233.00 ± 2.56	1.05 ± 0.04
	spr-04	18.57 ± 0.05	2.10 ± 0.25	6.74 ± 0.02	210.00 ± 3.58	0.86 ± 0.26
	sum-04	19.20 ± 0.33	1.44 ± 0.00	6.78 ± 0.02	310.00 ± 4.21	0.73 ± 0.12
	aut-04	18.50 ± 0.17	1.51 ± 0.01	6.70 ± 0.00	261.00 ± 1.25	0.47 ± 0.12
	win-05	18.60 ± 0.10	1.60 ± 0.02	6.56 ± 0.01	243.00 ± 1.00	0.60 ± 0.00
S50	sum-03	19.67 ± 0.05	1.80 ± 0.10	6.73 ± 0.06	178.27 ± 0.05	1.41 ± 0.14
	sum-04	19.57 ± 0.15	1.38 ± 0.05	7.14 ± 0.02	152.33 ± 0.57	1.25 ± 0.04
S56	sum-04	22.07 ± 0.11	0.86 ± 0.00	7.45 ± 0.03	21010.00 ± 149.33	6.00 ± 0.00
SO1	spr-03	18.05 ± 0.19	2.90 ± 0.00	6.94 ± 0.07	91.50 ± 0.01	0.49 ± 0.00
	sum-03	20.33 ± 0.15	2.33 ± 0.20	7.28 ± 0.09	153.00 ± 0.75	1.00 ± 0.09
	aut-03	18.63 ± 0.05	2.45 ± 0.06	7.36 ± 0.12	126.00 ± 0.02	0.54 ± 0.04
	spr-04	18.93 ± 0.05	2.10 ± 0.08	7.12 ± 0.02	99.00 ± 2.36	0.63 ± 0.04
	sum-04	14.60 ± 0.10	2.13 ± 0.01	6.87 ± 0.00	163.00 ± 3.69	0.63 ± 0.06
	sum-04	18.87 ± 0.15	1.79 ± 0.01	7.11 ± 0.01	135.00 ± 2.56	0.60 ± 0.00
	win-05	18.50 ± 0.20	2.20 ± 0.02	6.67 ± 0.01	136.00 ± 0.00	0.47 ± 0.11

Among the inorganic nitrogen forms, nitrate concentrations (mg L^{-1} $N\text{-}NO_3^-$) were higher than ammonium (mg L^{-1} $N\text{-}NH_4^+$) or nitrite concentrations (mg L^{-1} $N\text{-}NO_2^-$; data not shown) in most of the boreholes (Table 4.3). In some wells, nitrate concentrations were very high (Table 4.3). Soluble reactive phosphorus (SRP, mg L^{-1} $P\text{-}PO_4^{3-}$) and total phosphorus (TP, mg L^{-1} P) showed seasonal

concentrations of approx. 0.05 mg L^{-1} and 0.08 mg L^{-1} respectively (Table 4.3). Ferric iron usually displayed higher concentrations than ferrous iron. Total iron concentration ranged from 1 to 2 mg L^{-1} in most of the wells, and reached maximum values of approx. 20 mg L^{-1} (Table 4.3). Total alkalinity values were around 1 meq L^{-1} (Table 4.2).

Microbiological variables

Only prokaryotic cells were observed in microscope counts, with dominance of rod-shaped bacteria. No differences among cell morphologies were observed in samples from different depths or from different sites. Average bacterial abundance was $1.70 \times 10^7 \pm 1.99 \times 10^6$ bact mL^{-1} groundwater. Significant differences for bacterial abundance among piezometers were observed during the first three sampling campaigns in 2003 ($p \leq 0.039$). Seasonal differences for bacterial abundance in wells sampled more than three times were observed ($p \leq 0.047$) except in boreholes 39S2, 43S1, SO2a and SO2b ($p \geq 0.156$) (Figure 4.2, a and b). Bacterial abundances were statistically higher during summer or autumn 2003 than during winter or spring 2003 ($p \leq 0.042$), except for wells 43S1, SO2a, SO2b and SO1 ($p \geq 0.143$). During 2004, no statistical differences were observed among seasons ($p \geq 0.256$). This variable positively correlated with T ($r = 0.386$, $p = 0.001$, $n = 71$), nitrate ($r = 0.430$, $p = 0.000$, $n = 70$) and cell biomass ($r = 0.335$, $p = 0.004$, $r = 71$), whereas it negatively correlated with DO ($r = -0.237$, $p = 0.047$, $n = 71$), ferrous iron ($r = -0.316$, $p = 0.034$, $n = 41$) and total iron ($r = -0.265$, $p = 0.036$, $n = 63$). Bacterial abundances in wells S51, SO2a, SO2b and SO1 negatively correlated with rainfall from two months prior to sampling ($p \leq 0.046$). No correlations among bacterial abundance, screen depth, permeability and transmissivity were found, except during spring 2003, when bacterial abundance and depth positively correlated ($r = 0.890$, $p = 0.009$, $n = 8$).

Table 4.3 Chemical variables seasonally measured in the piezometers (mean ± SD) (SRP, soluble reactive phosphorus; win, winter; spr, spring; sum, summer; aut, autumn)

Piezometers	Seasons	Ammonium (mg L^{-1} N-NH_4^+)	Nitrate (mg L^{-1} N-NO_3^-)	SRP (mg L^{-1} P-PO_4^{3-})	Total P (mg L^{-1} P)	Ferric iron (mg L^{-1} Fe^{2+})	Ferrous iron (mg L^{-1} Fe^{3+})	Fe (mg L^{-1} Fe)
piso	sum-03	0.014 ± 0.008	1.009 ± 0.050	0.002 ± 0.001	0.057 ± 0.009	0.328 ± 0.198	0.917 ± 0.460	1.245 ± 0.654
	aut-03	0.048 ± 0.001	0.728 ± 0.032	0.016 ± 0.007	0.036 ± 0.007	0.082 ± 0.082	0.267 ± 0.065	0.349 ± 0.029
	sum-04	0.032 ± 0.013	1.313 ± 0.159	0.018 ± 0.004	0.019 ± 0.009	0.000 ± 0.000	0.349 ± 0.078	0.349 ± 0.078
	aut-04	0.493 ± 0.161	0.783 ± 0.072	0.013 ± 0.002	0.031 ± 0.002	0.000 ± 0.000	0.133 ± 0.027	0.133 ± 0.017
	win-05	0.024 ± 0.012	1.060 ± 0.108	0.011 ± 0.003	0.020 ± 0.006	0.073 ± 0.122	1.095 ± 0.914	1.167 ± 0.867
S51	win-03	0.152 ± 0.012	0.008 ± 0.001	0.047 ± 0.008	0.165 ± 0.003			
	spr-03	0.009 ± 0.000	0.059 ± 0.060	0.059 ± 0.004	0.286 ± 0.042	3.183 ± 0.746	2.510 ± 2.063	5.693 ± 5.810
	aut-03	0.065 ± 0.001	0.149 ± 0.007	0.046 ± 0.008	0.189 ± 0.096	0.272 ± 0.471	4.510 ± 1.666	4.327 ± 1.199
	spr-04	0.064 ± 0.022	0.118 ± 0.012	0.093 ± 0.008	0.353 ± 0.042	0.000 ± 0.000	1.954 ± 0.649	1.495 ± 0.649
	aut-04	0.045 ± 0.031	0.000 ± 0.000	0.120 ± 0.025	0.130 ± 0.017	0.000 ± 0.000	5.614 ± 1.329	5.614 ± 1.329
	win-05	0.006 ± 0.003	0.192 ± 0.007	0.239 ± 0.144	0.247 ± 0.025	0.080 ± 0.078	2.083 ± 1.948	2.163 ± 1.811
21S3	win-03	0.160 ± 0.103	0.008 ± 0.001	0.008 ± 0.000	0.042 ± 0.015			
	spr-03	0.010 ± 0.013	0.001 ± 0.004	0.000 ± 0.000	0.030 ± 0.001	0.700 ± 0.008	0.592 ± 0.129	1.292 ± 0.137
	sum-03	0.005 ± 0.004	0.054 ± 0.003	0.012 ± 0.006	0.040 ± 0.001	0.645 ± 0.037	0.509 ± 0.187	1.153 ± 0.533
	aut-03	0.044 ± 0.019	0.107 ± 0.003	0.011 ± 0.001	0.042 ± 0.006	0.048 ± 0.031	1.663 ± 0.205	1.712 ± 0.224
4S1	spr-03	0.016 ± 0.011	0.024 ± 0.033	0.001 ± 0.002	0.024 ± 0.005	2.030 ± 0.004	18.604 ± 2.200	20.635 ± 2.160
	sum-03	0.019 ± 0.012	0.031 ± 0.003	0.000 ± 0.000	0.043 ± 0.004	0.122 ± 0.040	0.654 ± 0.383	0.776 ± 0.343
	aut-03	0.047 ± 0.005	0.108 ± 0.004	0.003 ± 0.002	0.025 ± 0.008	0.000 ± 0.000	0.888 ± 0.158	0.888 ± 0.158
	aut-04	0.058 ± 0.002	0.248 ± 0.001	0.013 ± 0.002	0.062 ± 0.021	0.000 ± 0.000	1.647 ± 0.308	1.647 ± 0.308
	sum-04	0.038 ± 0.013	0.495 ± 0.025	0.006 ± 0.002	0.019 ± 0.011	0.119 ± 0.022	0.853 ± 0.046	0.972 ± 0.067
	aut-04	0.053 ± 0.037	0.004 ± 0.006	0.006 ± 0.001	0.028 ± 0.001	0.000 ± 0.000	1.655 ± 0.723	1.655 ± 0.723
	win-05	0.019 ± 0.018	0.346 ± 0.011	0.006 ± 0.001	0.007 ± 0.004	0.079 ± 0.068	2.928 ± 3.624	3.007 ± 0.004
L3	sum-03	0.029 ± 0.041	0.170 ± 0.158	0.004 ± 0.001	0.027 ± 0.000	1.024 ± 0.169	1.692 ± 1.575	2.715 ± 1.744
	spr-04	0.049 ± 0.014	0.112 ± 0.008	0.009 ± 0.005	0.083 ± 0.044	0.000 ± 0.000	1.702 ± 0.984	1.702 ± 0.984
L7	win-03	0.005 ± 0.007	0.069 ± 0.007	0.008 ± 0.001	0.031 ± 0.005			
	sum-03	0.000 ± 0.000	0.203 ± 0.016	0.007 ± 0.004	0.040 ± 0.017	0.658 ± 0.622	0.693 ± 0.600	1.351 ± 1.222
	sum-04	0.458 ± 0.016	0.204 ± 0.023	0.016 ± 0.007	0.087 ± 0.020	1.047 ± 0.753	12.910 ± 3.470	13.957 ± 2.784
	aut-04	0.309 ± 0.120	0.384 ± 0.114	0.026 ± 0.021	0.085 ± 0.007	0.000 ± 0.000	6.355 ± 1.105	6.255 ± 1.105
39S2	win-04	0.025 ± 0.009	1.678 ± 0.023	0.101 ± 0.004	0.144 ± 0.011	0.211 ± 0.366	0.806 ± 0.217	1.018 ± 0.254
	spr-04	0.064 ± 0.002	1.233 ± 0.132	0.041 ± 0.005	0.073 ± 0.003	0.002 ± 0.000	2.546 ± 1.647	2.567 ± 1.463
	sum-04	0.265 ± 0.035	2.077 ± 0.238	0.144 ± 0.054	0.156 ± 0.001	0.006 ± 0.002	0.186 ± 0.035	0.192 ± 0.032

Table 4.3 Continued

Piezometers	Seasons	Ammonium (mg L^{-1} $N\text{-}NH_4^+$)	Nitrate (mg L^{-1} $N\text{-}NO_3^-$)	SRP (mg L^{-1} $P\text{-}PO_4^{3-}$)	Total P (mg L^{-1} P)	Ferric iron (mg L^{-1} Fe^{2+})	Ferrous iron (mg L^{-1} Fe^{3+})	Fe (mg L^{-1} Fe)
39S2	aut-04	0.043 ± 0.015	1.028 ± 0.152	0.154 ± 0.071	0.155 ± 0.010	0.002 ± 0.003	0.884 ± 0.215	0.886 ± 0.212
	win-05	0.025 ± 0.005	1.112 ± 0.444	0.061 ± 0.008	0.075 ± 0.007	0.000 ± 0.000	2.114 ± 1.719	2.114 ± 1.719
TS4	win-03	0.156 ± 0.006	11.657 ± 0.931	0.003 ± 0.000	0.022 ± 0.009			
	sum-03	0.002 ± 0.000	9.245 ± 0.676	0.012 ± 0.006	0.013 ± 0.003	0.066 ± 0.032	0.364 ± 0.285	0.430 ± 0.317
TS4	aut-03	0.000 ± 0.000	12.568 ± 1.989	0.016 ± 0.006	0.022 ± 0.003	0.000 ± 0.000	0.602 ± 0.670	0.602 ± 0.671
	win-04	0.001 ± 0.003	11.645 ± 0.493	0.014 ± 0.006	0.059 ± 0.009	0.130 ± 0.125	0.376 ± 0.076	0.507 ± 0.245
43S1	win-03	0.078 ± 0.054	0.374 ± 0.054	0.016 ± 0.000	2.064 ± 0.063			
	spr-03	0.019 ± 0.000	0.866 ± 0.781	0.001 ± 0.001	0.040 ± 0.006	0.164 ± 0.039	1.185 ± 0.725	1.350 ± 0.764
	sum-03	0.003 ± 0.000	0.135 ± 0.008	0.006 ± 0.001	0.061 ± 0.031	0.357 ± 0.057	1.414 ± 0.899	1.771 ± 0.906
	aut-03	0.025 ± 0.017	0.464 ± 0.053	0.011 ± 0.003	0.062 ± 0.008	0.000 ± 0.000	2.392 ± 1.102	2.392 ± 1.102
S60	win-03	4.613 ± 0.019	0.060 ± 0.000	1.813 ± 0.014	1.814 ± 0.023			
	win-04	2.215 ± 0.411	0.348 ± 0.061	0.012 ± 0.007	0.073 ± 0.015	1.016 ± 1.001	0.747 ± 0.978	1.762 ± 2.086
	win-05	7.308 ± 1.175	0.160 ± 0.022	1.614 ± 0.062	1.640 ± 0.107	0.254 ± 0.298	1.066 ± 0.226	1.319 ± 0.511
TS2	win-03	0.136 ± 0.017	9.591 ± 2.141	0.028 ± 0.017	0.067 ± 0.041			
	sum-03	0.034 ± 0.037	12.226 ± 1.308	0.020 ± 0.004	0.065 ± 0.024	0.266 ± 0.175	0.741 ± 0.242	1.007 ± 0.139
	aut-03	0.031 ± 0.014	19.220 ± 0.284	0.008 ± 0.006	0.051 ± 0.016	0.000 ± 0.000	1.010 ± 0.297	1.010 ± 0.297
	win-04	0.000 ± 0.000	4.565 ± 0.294	0.041 ± 0.007	0.093 ± 0.011	0.136 ± 0.035	0.392 ± 0.068	0.528 ± 0.251
SO2a	spr-03	0.007 ± 0.001	0.780 ± 0.703	0.023 ± 0.003	0.024 ± 0.003	0.040 ± 0.021	0.361 ± 0.170	0.401 ± 0.191
	aut-03	0.010 ± 0.010	0.515 ± 0.065	0.038 ± 0.005	0.079 ± 0.012	0.003 ± 0.004	0.424 ± 0.082	0.427 ± 0.081
	spr-04	0.039 ± 0.013	0.717 ± 0.009	0.031 ± 0.002	0.058 ± 0.012	0.000 ± 0.000	1.018 ± 0.696	1.018 ± 0.696
	sum-04	0.000 ± 0.000	1.114 ± 0.095	0.033 ± 0.001	0.040 ± 0.008	0.005 ± 0.005	1.343 ± 0.696	1.348 ± 0.218
	aut-04	0.000 ± 0.000	0.411 ± 0.043	0.037 ± 0.002	0.053 ± 0.002	0.000 ± 0.000	0.263 ± 0.134	0.263 ± 0.134
	win-05	0.000 ± 0.000	0.535 ± 0.090	0.035 ± 0.001	0.037 ± 0.000	0.030 ± 0.029	0.620 ± 0.610	0.650 ± 0.591
4S2	win-03	0.273 ± 0.014	0.009 ± 0.000	0.009 ± 0.009	0.085 ± 0.009			
SO2b	spr-03	0.000 ± 0.000	0.671 ± 0.605	0.008 ± 0.001	0.015 ± 0.000	0.030 ± 0.005	0.328 ± 0.181	0.331 ± 0.176
	aut-03	0.017 ± 0.003	0.199 ± 0.005	0.016 ± 0.000	0.048 ± 0.009	0.000 ± 0.000	0.613 ± 0.571	0.613 ± 0.791
	spr-04	0.023 ± 0.011	0.334 ± 0.038	0.017 ± 0.003	0.022 ± 0.004	0.000 ± 0.000	0.503 ± 0.361	0.503 ± 0.361
	sum-04	0.027 ± 0.004	0.999 ± 0.011	0.015 ± 0.002	0.020 ± 0.005	0.004 ± 0.005	0.239 ± 0.080	0.242 ± 0.075
	aut-04	0.000 ± 0.000	0.222 ± 0.039	0.013 ± 0.002	0.044 ± 0.007	0.000 ± 0.000	0.077 ± 0.036	0.077 ± 0.036
	win-05	0.000 ± 0.000	0.250 ± 0.162	0.014 ± 0.000	0.015 ± 0.001	0.040 ± 0.054	0.546 ± 0.454	0.586 ± 0.437
S50	sum-03	0.000 ± 0.000	0.134 ± 0.001	0.022 ± 0.002	0.038 ± 0.011	0.029 ± 0.007	0.166 ± 0.030	0.195 ± 0.029
	sum-04	0.015 ± 0.001	0.261 ± 0.017	0.029 ± 0.000	0.033 ± 0.006	0.001 ± 0.000	0.408 ± 0.168	0.408 ± 0.168
S56	sum-04	7.728 ± 1.385	0.431 ± 0.035	0.423 ± 0.041	0.521 ± 0.041	0.123 ± 0.083	1.317 ± 0.725	1.440 ± 0.649
SO1	spr-03	0.000 ± 0.000	0.622 ± 0.562	0.007 ± 0.000	0.026 ± 0.004	0.000 ± 0.000	0.232 ± 0.067	0.232 ± 0.067
	sum-03	0.034 ± 0.029	0.223 ± 0.019	0.028 ± 0.003	0.032 ± 0.005	0.425 ± 0.197	1.177 ± 0.655	1.602 ± 0.725
	aut-03	0.031 ± 0.007	0.571 ± 0.020	0.023 ± 0.002	0.040 ± 0.014	0.000 ± 0.000	5.832 ± 4.963	5.832 ± 8.963
	spr-04	0.038 ± 0.012	0.466 ± 0.049	0.019 ± 0.002	0.031 ± 0.011	0.000 ± 0.000	0.318 ± 0.178	0.318 ± 0.178
	sum-04	0.000 ± 0.000	0.879 ± 0.083	0.025 ± 0.001	0.035 ± 0.007	0.011 ± 0.005	0.158 ± 0.018	0.169 ± 0.023
	sum-04	0.023 ± 0.018	0.540 ± 0.024	0.027 ± 0.009	0.045 ± 0.008	0.000 ± 0.000	0.227 ± 0.026	0.227 ± 0.026
	win-05	0.000 ± 0.000	0.151 ± 0.009	0.024 ± 0.002	0.034 ± 0.003	0.018 ± 0.032	0.478 ± 0.441	0.496 ± 0.426

Mean cell biomass was determined to be 77 ± 32 fgC (Figure 4.2, c and d). Cell biomass showed significant differences among piezometers during all seasons ($p \leq 0.048$), except in winter 2005 ($p \geq 0.109$). Significant seasonal differences for cell biomass were observed in piezometers sampled more than three times during the study period ($p = 0.000$), except in well TS4 ($p = 0.204$). A significant increase in cell biomass during 2003 was observed toward the warmer seasons in all piezometers ($p \leq 0.004$), except in wells TS4 and SO2a ($p \geq 0.654$). A significant, although less evident, increase in cell biomass throughout 2004 was observed in wells S51, 4S1, SO2a, SO2b and SO1, with statistical differences between winter or spring and summer or autumn ($p \leq 0.023$). Cell biomass positively correlated with T ($r = 0.487$, $p = 0.000$, $n = 71$) and negatively with DO ($r = -0.242$, $p = 0.037$, $n = 71$). In wells S51 and SO1 cell biomass negatively correlated with rainfall from two months prior to sampling ($p \leq 0.028$). No significant correlations were found among cell biomass, depth, permeability and transmissivity in any season.

Average bacterial biomass was 1.40 ± 0.57 μgC mL^{-1}. Differences in the means of bacterial biomass among piezometers were statistically significant in all seasons ($p = 0.000$), but more evident during 2003 than during 2004. Seasonal differences were observed in all piezometers sampled more than three times throughout the study period ($p = 0.000$) (Figure 4.2, e and f). A significant increase in bacterial biomass was seasonally observed during 2003 in all wells, with higher values during summer or autumn than during winter or spring ($p \leq 0.008$), except in borehole

SO2a ($p \geq 0.378$). During 2004, a similar temporal pattern was observed ($p \leq 0.023$), except in well SO2a ($p \geq 0.567$). Bacterial biomass positively correlated with T ($r = 0.471$, $p = 0.000$, $n = 71$) and nitrate ($r = 0.423$, $p = 0.000$, $n = 71$), and negatively with DO ($r = -0.276$, $p = 0.021$, $n = 71$). In wells S51, SO2b and SO1 bacterial biomass negatively correlated with rainfall from two months prior to sampling ($p \leq 0.035$). This variable did not correlate with depth, permeability or transmissivity in any season.

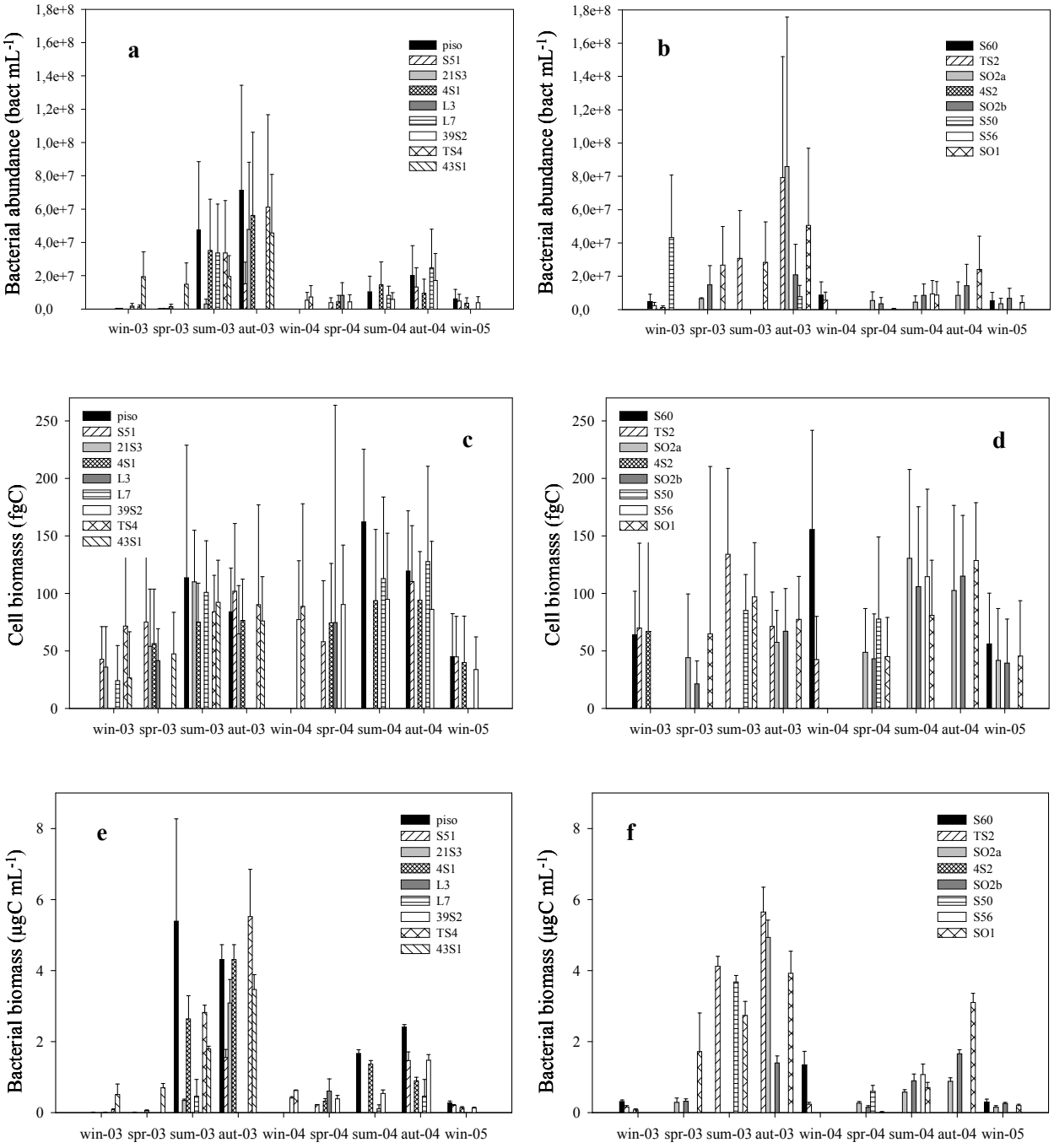


Figure 4.2 Seasonal changes in microbiological variables during the study period. Bacterial biomass (a and b), cell biomass (c and d), bacterial biomass (e and f) (win, winter; spr, spring; sum, summer; aut, autumn; solid bars denote standard deviation).

Microbial activities of functional groups, except those for nitrifying bacteria (NB), were found in this aquifer system. Significant differences for the activities of the different functional groups, measured as the mean of the first day after incubation in which activity was detected, were observed in all seasons ($F \geq 5.530$, $p \leq 0.009$) (Table 4.4). During 2003 and winter 2004, IRB showed statistically higher activities than SRB and DNB ($p \leq 0.001$), whose activities were not significantly different ($p \geq 0.099$). In summer 2004, IRB and SRB showed statistically similar activities ($p \geq 0.123$) and significantly higher than DNB ($p \leq 0.009$). During 2003, higher activities in summer than in winter were found in all cases, although they were only significant for IRB and SRB activities in some wells (21S3, L7 and TS2; $p \leq 0.035$). In general, there were not significant differences in the means of microbial activities between summer and winter during 2004. No significant correlations were found among microbial activities, depth, permeability and transmissivity.

Table 4.4 Summary of microbial activities for different functional groups provided by BART™ tests (DNB denitrifying bacteria; IRB, iron-related bacteria; SRB, sulphate-reducing bacteria; win, winter; sum, summer). Numbers indicate first day in which bacterial activity was recorded

Piezometers	DNB				IRB				SRB			
	win-03	sum-03	win-04	sum-04	win-03	sum-03	win-04	sum-04	win-03	sum-03	win-04	sum-04
piso		10		3		4		2		4		9
S51	6				3				10			
21S3	5	3			10	4			10	10		
4S1		9		2		2		3		5		3
L7	10	10		2	4	3		2	7	5		7
39S2			10	10			3	2			9	4
TS4	8	4	4		4	3	4		10	6	10	
43S1		10				4				6		
S60	5		4		1		1		3		4	
TS2	4	4	4		3	1	4		8	5	8	
SO2a				3				3				6
4S2	7				4				10			
SO2b				3				2				4
S50		6				2				8		
S56				1				2				5
SO1		10		6		3		10		5		3

Exploratory statistical analyses

Bartlett´s χ^2 test showed that the two sets of variables (microbiological and physicochemical) in the canonical correlation analysis were not independent ($\chi^2 = 60.985$, df = 39, p = 0.014); the first two canonical variate pairs were significantly correlated (Table 4.5). The strong correlation observed for the first canonical variate pair symbolizes a correlation between a variate that combines T, DO, nitrate, ferrous iron and total iron, and another variate that integrates bacterial abundance, cell biomass and bacterial biomass (Figure 4.3). Bacterial abundances, and mainly, cell and bacterial biomasses increase with higher temperatures and nitrate concentrations but, with higher DO, ferrous ion and total iron concentrations, bacterial abundances, cell biomasses and bacterial biomasses show a decrease. The correlation observed for the second canonical variate pair was less significant (Table 4.5). The variance explained by the first three factors included in the principal component analysis of the correlation matrix performed was 70.21%. Microbiological variables, temperature and nitrate concentration defined the principal component I. Samples were classified into two major

groups in relation to axis I: one on the positive side (winter/spring 2003, winter/spring 2004, and winter 2005) and another on the negative side (summer/autumn 2003, and summer/autumn 2004) (Figure 4.4).

Table 4.5 Results after Bartlett´s χ^2 test with successive variate pairs removed for the canonical correlation analysis

Variate pair removed	Canonical R	Canonical R^2	χ^2	df	p
0	0.710	0.504	80.709	39	0.000
1	0.557	0.310	37.534	24	0.039
2	0.461	0.212	14.673	11	0.198

DISCUSSION

Physicochemical variables

As surface waters, whose temperature and dissolved oxygen values display temporal patterns (Coleto, 2003), groundwater temperature (T) and dissolved oxygen (DO) also exhibited temporal patterns in the aquifer system of Doñana. In other aquifer systems, no significant seasonal differences for T, at least between winter and summer, were observed (Balkwill *et al.*, 1989; Pedersen *et al.*, 1996). This aquifer system therefore appears to be very different to other subsurface systems considered to be highly stable (Vorobyova *et al.*, 1997). The decrease in groundwater DO from winter to autumn during both years might be due to inorganic reactions involving reduced metals, microbial utilization of reduced organic matter, or both (Phelps *et al.*, 1994; Brugger *et al.*, 2001; López-Archilla *et al.*, 2007). Organic matter concentration in the Doñana aquifer is moderately high (Coleto, 2003); as a result, low DO concentrations found in autumns might be due to microbial consumption (Griebler *et al.*, 2002). Electric conductivity (EC) was higher in wells located close to the marsh because there is a clear influence of brackish water. pH was close to neutral and, due to the absence of correlations between this chemical variable and the microbiological variables, we suggest that pH was not a key factor controlling the spatiotemporal distribution of microbial communities present in this aquifer system, as has been previously pointed out (Gounot, 1996); however, pH was a central factor controlling the diversity and the taxonomic composition of microbial communities in Doñana's groundwater (López-Archilla *et al.*, 2007).

Although aquifers have often been considered to be oligotrophic environments (Mikell *et al.*, 1996) more similar to open marine systems than to lakes or shallow lakes, and with bacterial densities close to those observed for oceans (Pedersen and Ekendahl, 1990), we consider that these ideas can neither be generalized nor extrapolated. Doñana's aquifer system, at least its upper unit, appears to be meso- or eutrophic if concentrations of nitrogen and phosphorus inorganic chemical forms are considered (Table 4.3). Although several wells were used in this study, and spatiotemporal variability is notable, ammonium, nitrate, soluble reactive phosphorus (SRP) and total phosphorus (TP) concentrations were higher than, or at least similar to, those values reported for other aquifer systems (Alfreider *et al.*, 1997; Murphy and Schramke, 1998; Trojan *et al.*, 2003; Mehnert *et al.*, 2007). Most of the wells in this study showed higher concentrations for nitrate than for ammonium, probably because most of them were well oxygenated during the study period

(Tables 4.2 and 4.3). Nitrate concentrations are usually low in groundwaters, unless there is a pollution source from agricultural fertilizers (Gounot, 1996). In this study, high nitrate concentrations found in wells TS2 and TS4 are explained by the presence of intensive strawberry crops (Manzano *et al.*, 2007). A high iron concentration in the groundwater is explained by the presence of an intermediate layer with iron oxide minerals (Trick and Custodio, 2004). High total iron concentrations, as well as ferrous and ferric iron concentrations, had a considerable influence on the type of microorganisms found in this ecosystem (López-Archilla *et al.*, 2007). At the same time, high concentrations of organic matter, that can act as a source of electrons for microorganisms, were also indirectly determined in this aquifer system (Coleto, 2003). Both ferric iron and nitrate can act as electron acceptors when dissolved oxygen is depleted. In fact, iron-related bacteria (IRB) have displayed the highest activities, highlighting the relative importance of iron (and its chemical forms) in the general microbial metabolism of this aquifer system. Although high nitrate concentrations were found in this aquifer system, however, denitrifying bacteria (DNB) exhibited lower activity levels than IRB and sulphate-reducing bacteria (SRB), demonstrating that there are no reasons to consider linear relationships among certain chemical forms, used as electron acceptors by microorganisms, and functional groups (Mauck and Roberts, 2007), because aquifer heterogeneity results in a heterogenerous distribution of the microbial communities and their activities (Goldscheider *et al.*, 2006). In any case, the activity of microbial communities is probably influencing the geochemistry of this aquifer system, as has been observed elsewhere (Bennett *et al.*, 2000; Chapelle, 2000; Penny *et al.*, 2003; Dassonville *et al.*, 2004; Haack *et al.*, 2004; Roadcap *et al.*, 2006).

Microbiological variables

Prokaryotes appear to dominate the microbial communities in this aquifer system. Eukaryotes were not detected in microscopic counts. This is not surprising because, although the presence of algae, protozoa and fungi could be important in some aquifer ecosystems, prokaryotes represent, by far, the most abundant and diverse microbial group in aquifers, at least in the phreatic zone (Balkwill, 1989; Sinclair and Ghiorse, 1989; Whitman *et al.*, 1998).

Short rod-shaped bacteria have some advantages over large rod-shaped and filamentous bacteria for transport through sandy sediments (Harvey *et al.*, 1984). Data in this study confirm previous ones (Velasco Ayuso *et al*., 2008) and demonstrate that mean bacterial abundance observed in this aquifer system was, at least, one order of magnitude higher than that found in other sedimentary and relatively similar aquifer ecosystems (Harvey *et al.*, 1984; Kölbel-Boelke *et al.*, 1988; Marxsen, 1988; Hirsch and Rades-Rohkohl, 1990; Hazen *et al.*, 1991; Alfreider *et al.*, 1997; Griebler *et al.*, 2002). Groundwater mean bacterial abundance found in this study was also higher than planktonic bacterial densities found in granite (Eydal and Pedersen, 2007) or rock aquifer systems (Lehman *et al.*, 2004). Indeed, planktonic bacterial abundance in the Doñana aquifer was close to values reported for attached bacteria in other sedimentary aquifer systems (Alfreider *et al.*, 1997; Kieft *et al.*, 1998; Martino *et al.*, 1998). Several comparative studies have shown that attached bacteria exhibit more density than unattached bacteria in sedimentary aquifers (Alfreider *et*

al., 1997), as well as in rock aquifers (Lehman *et al.*, 2004). Bearing in mind that our data only show the density of planktonic bacteria, the real bacterial abundance of Doñana's aquifer system might well be higher. However, the abundance of unattached bacteria can be significant in shallow, eutrophic, and sedimentary aquifer systems (Harvey *et al.*, 1984; Harvey and George, 1987; Bengtsson, 1989; Griebler *et al.*, 2002).

Average cell biomass values were similar to those found in other sandy sediments (Bone and Balkwill, 1988), but were slightly higher than a value reported for a sedimentary, sandy, and very similar aquifer system (Marxsen, 1988). Bacterial biomass values were in the same order of magnitude as those found in other sandy, aquifer systems (Marxsen, 1988).

Distribution of microbial communities in the aquifer system

Differences in bacterial abundance among wells sampled during the same season were not significant in most sampling campaigns, as has been observed elsewhere (Kölbel-Boelke *et al.*, 1988). Moreover, differences for cell and bacterial biomass among wells were not great during some seasons. In sandy aquifer systems, spatial heterogeneity can be more important for sediments than for groundwaters on a small scale (Fredrickson *et al.*, 1991; Brockman and Murray, 1997), in spite of the homogeneity of lithologies that can occur on a greater scale (Zhou *et al.*, 2004). Consequently, microbiological variables could present fewer and less significant differences among groundwater samples than among sediment core samples (Kölbel-Boelke *et al.*, 1988). Moreover, differences among replicates of the same aquifer sample point can be higher than differences among different aquifer sample points (Brockman and Murray, 1997). In other studies, no differences in bacterial abundance were found, either in groundwaters (Pedersen and Ekendahl, 1990) or in sediment core samples (Hazen *et al.*, 1991). Finally, it should be borne in mind that field samples represent the summation of a complex series of environmental interactions acting over vastly different temporal and spatial scales, being difficult to find clear spatiotemporal patterns in microbial communities (Shi *et al.*, 1999).

Although it was presumed that bacterial abundance decreases with depth, in general, no obvious correlations between these variables have been found in shallow, sedimentary aquifer systems (Beloin *et al*., 1988; Sinclair and Ghiorse, 1989; Phelps *et al*., 1994; Fredrickson *et al*., 1997; Martino *et al*., 1998). Negative relationships have been observed, however, between these two variables in the vadose zone of some low recharge aquifer systems (Fredrickson *et al*., 1997; Kieft *et al*., 1998). In Doñana's aquifer system, no significant correlations were detected between depth and bacterial abundance during any season, with exceptions in some deep wells. Moreover, no significant correlations were found between cell biomass and depth during any season in the Doñana aquifer system. Consequently, depth does not seem to be a key factor controlling microbial communities in terms of abundance and biomass in this aquifer system, at least in the range of depths studied. Furthermore, no significant correlations were found between microbial functional groups and depth, although conclusions should be taken with care because the number of samples was low ($n \leq 8$).

Grain size has often been considered to be one of the most important factors controlling microbial abundance and activity in aquifer systems (Musslewhite *et al.*, 2003). Layers with higher clay content usually exhibit lower attached bacterial abundances than sandy layers (Fredrickson *et al.*, 1997; Musslewhite *et al.*, 2003), although positive correlations between sediment clay content and bacterial density have also been observed (Balkwill, 1989), showing the need to consider site-specific environmental factors in order to understand microbial distribution and activity (Kieft *et al.*, 1998). In spite of the fact that subsurface sediments and groundwaters represent different milieus (Madsen and Ghiorse, 1993), grain size might also affect the planktonic microbial communities because there is a permanent cell exchange between suspended and attached bacteria (Hirsch and Rades-Rohkohl, 1990; Madsen and Ghiorse, 1993). In the aquifer system of Doñana, piezometers with the finest materials (high clay contents) in the screen region (TS2 and 4S2) (Table 4.1) showed lower bacterial abundances than boreholes with coarser materials (medium sands or coarse sands), although no significant differences were found. Moreover, wells 21S3 and S51, with fine sands in the screen region, showed lower bacterial densities, often with significant differences, than other piezometers with coarser lithologies (Figure 4.2, a and b) and borehole L7, with coarse sands in the screen region, displayed high bacterial abundances during three sampling seasons. Consequently, a clearer relationship between grain size and bacterial abundance than between depth and bacterial abundance was observed in the Doñana aquifer system. Nonetheless, differences for cell biomasses between wells with fine materials and wells with coarse materials were less evident. Finally, no significant or clear patterns were found between the microbial activities of functional groups and grain size, although conclusions should be reached again with care because only a few samples were considered ($n \leq 5$).

The apparent control of grain size over microbial communities might be due to some hydrogeological parameters, such as permeability, porosity or transmissivity (Brockman and Murray, 1997; Musslewhite *et al.*, 2003). Areas that show higher hydraulic conductivities tend to display higher bacterial biomasses (Fredrickson *et al.*, 2004) and activities (Chapelle and Lovley, 1990), because this variable determines the hydrological flows and, consequently, the nutrient supplies to bacteria (Lehman *et al.*, 2001; Zhou *et al.*, 2004) and the movement of cells through the aquifer system (Balkwill *et al.*, 1998). It seems that clay content, *per se*, may not directly control microbial population densities in the subsurface, but rather the influence of clay on microbial populations in the subsurface may be due to the effect that clay has on hydraulic conductivity or water activity (Fredrickson *et al.*, 1991). However, no correlations between microbiological and hydrogeological variables (permeability and transmissivity) were observed in this study. Considering the grain size homogeneity reported at least for the upper unit of this aquifer system (Trick and Custodio, 2004), where most of the sampled piezometers are located, a detailed hydrogeological study would contribute to clarifying the spatial distribution of their microbial communities. A common project between microbiologists and hydrogeologists is desirable, but only if both share the same spatiotemporal scale (Brockman and Murray, 1997).

Moreover, and taking into account the correlations found between microbiological variables and rainfall, hydrology, determined by rainfall, could exert an important control over the microbial

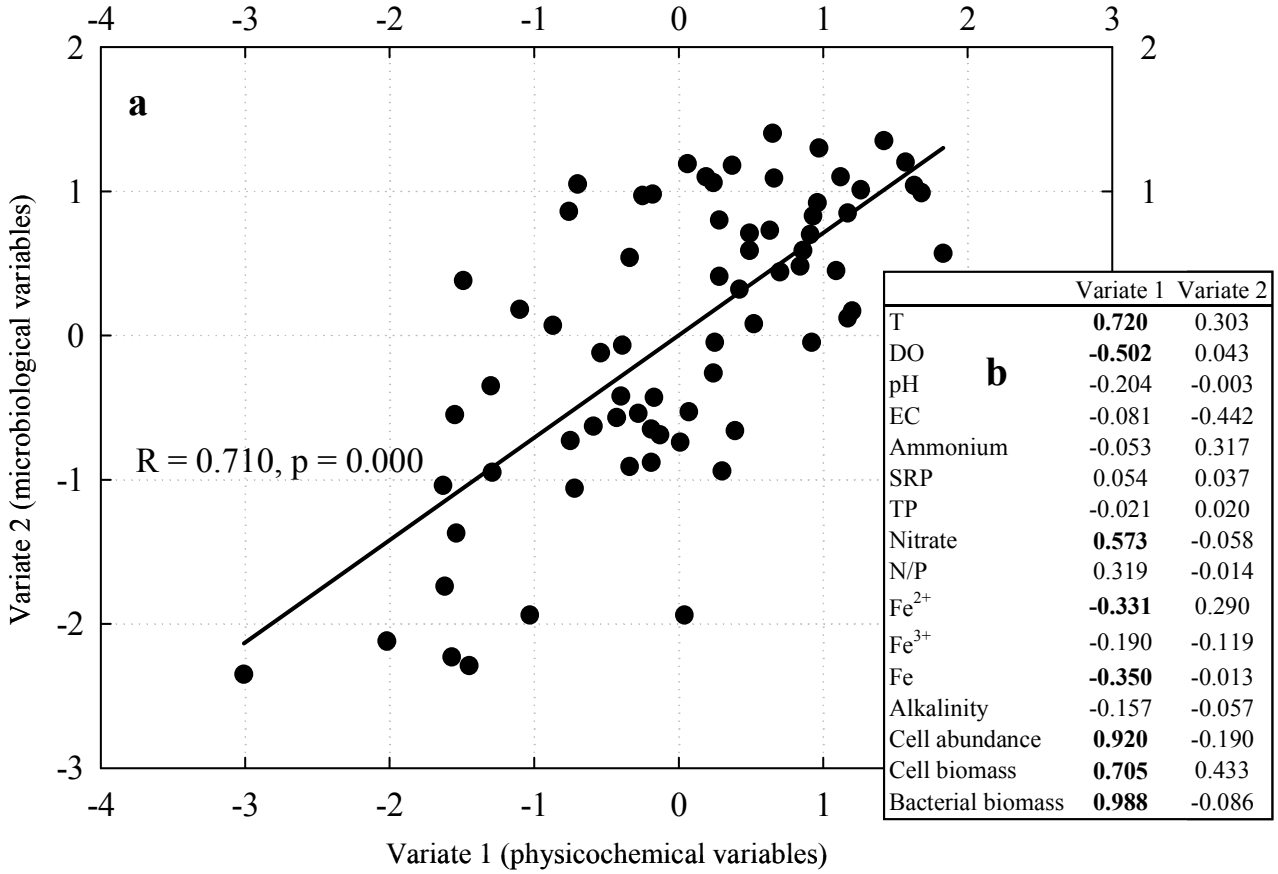
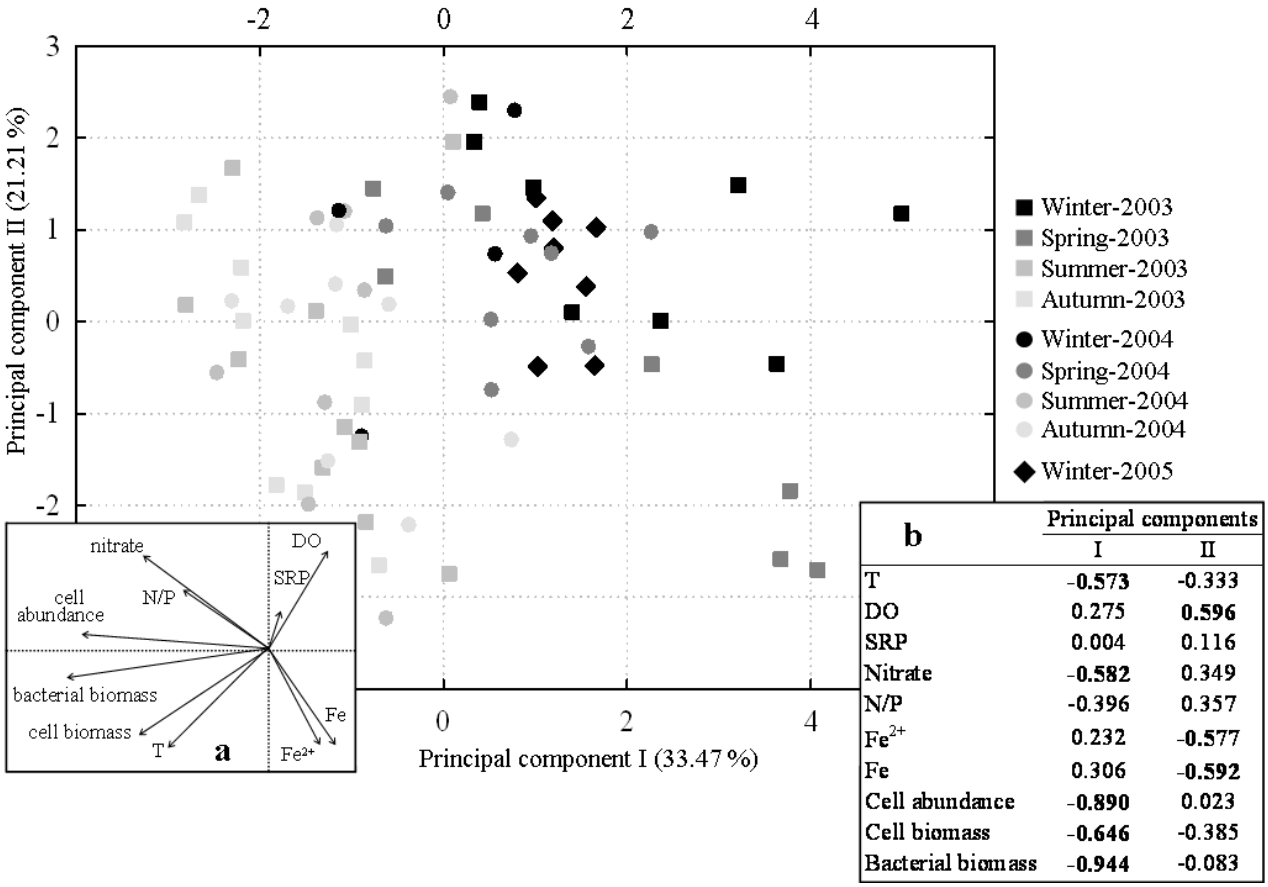


Figure 4.3 Canonical correlation analysis showing physicochemical variables *vs* microbiological variables form the first variate pair (a). Contributions of the descriptors to the first variate pair (b) (boldface type indicates the major canonical loadings in the first variate pair) (T, temperature; DO, dissolved oxygen; EC, electric conductivity; SRP, soluble reactive phosphorus; TP, total phosphorus).

communities of this aquifer system, mainly if rainfall is considered to be the only source of freshwater in the aquifer system and, as a result, largely determines hydrogeological flows and phreatic levels (Trick and Custodio, 2004). Bacterial abundance and cell biomass negatively correlated with rainfall two months prior to sampling in wells SO1, SO2a, SO2b and S51. Curiously, these wells are located close to the lower unit, where important hydrological horizontal flows occur (SO1, SO2a and SO2b), or close to the ecotone, the most important discharge area of the aquifer system (S51). As a consequence, it seems that hydrology has a relatively significant influence over microbial communities in areas where regional hydrogeological flows are significant. However, other wells located in the ecotone (21S3) or close to other important discharge areas of the aquifer (4S1, 4S2 and L3) did not correlate with rainfall, which suggests that there are other variables, probably also related to hydrogeology, controlling the distribution of microbial communities in this aquifer system.

Significant correlations between microbiological and physicochemical variables in sedimentary aquifers (Martino *et al.*, 1998; Musslewhite *et al.*, 2003; Santoro *et al.*, 2006) are scarce, because aquifer microbial communities are controlled by environmental attributes that are more difficult to quantify, such as microscale spatial heterogeneity and temporal variability of

geochemical parameters (Kölbel-Boelke *et al.*, 1988; Fredrickson *et al.*, 1991; Brockman and Murray, 1997; Santoro *et al.*, 2006; Mauck and Roberts, 2007). However, some correlations have been observed between groundwater physicochemical variables and microbial communities in crystalline rock aquifers (Pedersen and Ekendahl, 1990). In the present study, a canonical correlation analysis showed a strong correlation between a variate integrating nitrate, ferrous iron and total iron and a variate combining bacterial abundance and both cell and bacterial biomass (Figure 4.3). Nitrate concentrations are usually low in aquifers unless a source from agricultural fertilizers exists (Gounot, 1996; Brugger *et al.*, 2001), and this is the case observed for some piezometers (Tables 4.1 and 4.3) located in the vicinity of strawberry crops in Doñana. Indeed, three out of five piezometers affected by high nitrate concentrations (TS2, TS4 and L7) showed high or the highest bacterial abundance and cell biomass values during some seasons. Relationships among microbiological variables, ferrous iron and total iron were not so clear, but the ferric iron might probably be used as an electron acceptor when dissolved oxygen is depleted (McLean *et al.*, 2006).



b

	Principal components I	II
T	**-0.573**	-0.333
DO	0.275	**0.596**
SRP	0.004	0.116
Nitrate	**-0.582**	0.349
N/P	-0.396	0.357
Fe^{2+}	0.232	**-0.577**
Fe	0.306	**-0.592**
Cell abundance	**-0.890**	0.023
Cell biomass	**-0.646**	-0.385
Bacterial biomass	**-0.944**	-0.083

Figure 4.4 Positions of the 71 seasonal samples plotted in the reduced space of the first two principal components, showing both the ten descriptors or variables projected on the plane determined by the first two principal axes (box a) and the factor loadings of these descriptors on each of the first two axes (box b) (boldface type indicates the major factor loadings on each axis) (T, temperature; DO, dissolved oxygen; SRP, soluble reactive phosphorus).

Temporal pattern

A temporal pattern was observed in the microbial communities of this aquifer system, mainly during 2003, but also during 2004, with highest bacterial abundances, cell biomasses and bacterial biomasses during the warmest months of the year (Figure 4.4). Similar results were observed in a parallel study (Velasco Ayuso *et al*., 2008). Temperature correlated with these three microbiological variables and seemed to be the most important physical factor controlling them throughout the year. Light increases of this variable triggered microbiological processes, including functional groups. However, the temporal pattern was not clear in deep wells (SO1, SO2a and SO2b). As a consequence, in deep areas of Doñana's aquifer system there are probably other factors, perhaps more related to hydrogeology than to temperature, controlling the temporal distribution of the microbial communities. Seasonal variations of microbial communities in other aquifer systems have also been described (Balkwill and Ghiorse, 1985; Beloin *et al*., 1988; Bone and Balkwill, 1988).

CONCLUSIONS

Although it would be incorrect to depict general conclusions if only a limited number of samples in a large system were analyzed, this study has demonstrated the presence of important and active microbial communities, in terms of bacterial abundance, cell biomass and functional groups, in an area encompassing approximately 100 km^2 of the aquifer system located in Doñana, corroborating parallel results obtained in another study conducted in a smaller area. Due to the homogeneity, not only for the groundwater but also for the lithologies, the spatial pattern of the unattached community was difficult to explain. Among the abiotic factors that usually control the spatial distribution, depth did not play any role, at least in the studied range, and grain size seemed to exert a moderate control on bacterial abundance, although less clear on cell biomass. It is, however, plausible that hydrogeology, through some other related variables such as permeability, porosity or transmissivity, plays a more important role, controlling the spatial pattern of microbial communities, although unfortunately, there are no hydrogeological models with adequate scales for groundwater microbiology studies. Moreover, nitrate seemed to play an important role in controlling bacterial abundance and biomass, because the higher the nitrate concentration, the higher the bacterial abundance, cell biomass and bacterial biomass. On the other hand, all boreholes (except wells SO1, SO2a and SO2b) sampled more than three times throughout the study period showed a clear temporal pattern, mainly during 2003, with higher bacterial abundances, cell biomasses and bacterial biomasses during summers and autumns than during springs and winters, showing the microbial communities of this aquifer system to be highly reactive. Temperature was the most important factor controlling this temporal pattern, triggering not only abundance and carbon content but also activity, mainly IRB activity. As a result, when referring to microbial ecology in the aquifer system of Doñana, there is a need to talk of a dynamic system, probably more similar to the surface aquatic systems located in the same area than to other aquifers. Finally, hydrogeological models jointly developed between microbiologists and hydrogeologists will provide an understanding of the control exerted by hydrology (rainfall, hydrogeological flows,

hydrological relationships between groundwater and surface aquatic systems) on the microbial communities of this aquifer system, a phenomenon that has been partially described in this study.

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CAPÍTULO 5. La función de las comunidades microbianas del sistema acuífero de Doñana I: producción bacteriana de carbono

5. MICROBIAL ACTIVITIES IN A COASTAL, SANDY AQUIFER SYSTEM (DOÑANA NATURAL PROTECTED AREA, SW SPAIN)

Geomicrobiology Journal (2010), 27, 409-423

ABSTRACT

We conducted a seasonal research of the activities of microbial communities in a coastal, sandy aquifer system located in the Doñana Natural Protected Area (SW, Spain). Groundwaters from 30 piezometers were sampled over a two-year period. The proportion of active microbial biomass ranged from 0.02 to 6.36% of the total microbial biomass, while the active microbial biomass ranged from $7.42 \times 10^{-3} \pm 2.20 \times 10^{-4}$ to 17.30 ± 3.71 ngC mL^{-1}. Bacterial carbon production, measured through the incorporation of [^{3}H]leucine into cellular proteins, showed a mean value of 0.18 ± 0.72 ngC mL^{-1} h^{-1} in all wells and all seasons. Bacterial growth rates ranged from 0.03 to 87.26 days. These activities exhibited spatiotemporal patterns. Temperature and the presence of nutrients and organic matter appear to be important factors controlling these patterns. However, hydrogeological flows, both local and regional, seemed to constitute the most important factor determining these spatiotemporal patterns, probably because the distribution of nutrients in aquifer systems is mainly controlled by these hydrogeological flows. The well-known hydrological flows connecting surface waters and groundwaters in Doñana support the assumption that both water compartments form a unique entity (called *hydroecosystem*), which functions as a whole. Consequently, not only microbial processes in surface waters can influence ecological processes in groundwaters, the characteristics of surface waters can also be affected by groundwater chemical processes, among others, mediated by the activities of microbial communities.

5. ACTIVIDADES MICROBIANAS EN UN SISTEMA ACUÍFERO SEDIMENTARIO Y COSTERO (ÁREA NATURAL PROTEGIDA DE DOÑANA, SW ESPAÑA)

Geomicrobiology Journal (2010), 27, 409-423

RESUMEN

Con el objetivo de conocer las actividades de las comunidades microbianas que habitan en el sistema acuífero costero y arenoso del Área Natural Protegida de Doñana (suroeste de España), sus aguas subterráneas fueron muestreadas en 30 piezómetros a lo largo de dos años. La proporción de biomasa microbiana activa osciló entre un 0.02 y un 6.36% del total de biomasa microbiana, mientras que la biomasa microbiana activa varió entre $7.42 \times 10^{-3} \pm 2.20 \times 10^{-4}$ y 17.30 ± 3.71 ngC mL^{-1}. La producción bacteriana de carbono, estimada a través de la incorporación de leucina[^{3}H] en las proteínas celulares, mostró un valor medio de 0.18 ± 0.72 ng mL^{-1} h^{-1}, teniendo en cuenta todos los piezómetros y todas las estaciones. Las tasas de eficiencia de crecimiento bacteriano variaron entre 0.03 y 87.26 días. Las actividades de las comunidades microbianas en este acuífero mostraron patrones espaciotemporales. La temperatura y los nutrientes parecieron ser factores importantes que ejercen un control sobre estos patrones. Sin embargo, los flujos hidrológicos, tanto locales como regionales, son los que realmente controlan estos patrones espaciotemporales, probablemente porque la distribución de los nutrientes en los sistemas acuíferos está determinada principalmente por estos flujos hidrológicos. Las conexiones hidrológicas entre las aguas subterráneas y las aguas superficiales en Doñana permiten pensar que ambos compartimentos hídricos forman una única entidad (llamada *hidroecosistema*) que funciona como un todo. Como consecuencia, parece que no solamente los procesos microbianos que se desarrollan en las aguas superficiales pueden influir en los procesos ecológicos de las aguas subterráneas, sino que las características de las aguas superficiales, y también sus procesos ecológicos, pueden verse afectadas por procesos químicos que se llevan a cabo en el acuífero, mediados, entre otros, por la actividad de las comunidades microbianas que aquí habitan.

INTRODUCTION

The study of life beneath the surface of the Earth was of relatively minor scientific interest prior to the 1970s (Ghiorse and Wilson, 1988; Madsen and Ghiorse, 1993). Until recently most people, including microbial ecologists, had never thought about it seriously. Indeed, the aphorism "*out of sight, out of mind*" aptly describes a traditional view of the terrestrial subsurface held by generations of human beings (Ghiorse and Wilson, 1988). In fact, during decades, it was widely assumed that, even shallow aquifers, *i.e.*, the areas below the root zone of plants, were sterile habitats (Chapelle, 2001). The little attention paid by the scientific community to aquifer systems has been largely due to their inaccessibility and the difficulties involved in obtaining uncontaminated, representative samples (Cullimore, 1993). It is now accepted, however, that microbial communities are widespread in the Earth's subsurface systems, and that they appear to be metabolically and phylogenetically more diverse than previously thought (Griebler and Lueders, 2009), even in deep geological formations (Hallbeck and Pedersen, 2008; Pedersen *et al*., 2008). Currently, aquifer systems are considered as ecological systems, with their inhabitants as members of complex communities, rather than just aseptic, inner reservoirs of water for human consumption (Goldscheider *et al*., 2006; Humphreys, 2009).

Microbial communities are complex and diverse assemblages of species presenting different morphologies and metabolic characteristics. A major question in microbial ecology is to determine the importance of some microorganisms in the activity and productivity reflected by the whole microbial community in the ecosystem functioning (Servais *et al*., 2003). Bacteria, among other microorganisms, are fundamental to the structure and dynamics of nutrient cycling and energy fluxes within all ecosystems (Wetzel, 2001). In order to establish the ecological importance of microbial communities in an ecosystem, the active microbial biomass, the bacterial carbon production and the bacterial growth rate are, among others, fundamental variables (Bååth, 1994). The active microbial biomass, when used in conjunction with estimates of productivity, can be applied to place limitations on material and energy fluxes through aquatic microbial food webs (Karl, 1993). Determination of bacterial carbon production is fundamental to an evaluation of the role of microorganisms in food webs (Marxsen, 1996; Fischer and Pusch, 1999) and important with regard to quantifying the contribution of bacteria to carbon cycling in ecosystems (Buesing and Gessner, 2003). Estimation of bacterial growth rates helps to describe the physiological state of subsurface microflora and provides an understanding of natural microbial communities in aquifer systems (Thorn and Ventullo, 1988).

Microbial activities and metabolism pathways in aquifer systems have been partially depicted by several studies. However, studies performed in sedimentary or sandy, relatively shallow aquifers, although moderately abundant (Alfreider *et al*., 1997; Martino *et al*., 1998; Zhou *et al*., 2004; Tietz *et al*., 2007), are more scarce than those reported for crystalline or rock, deep aquifer systems (Fredrickson and Balkwill, 2006; Eydal and Pedersen, 2007; Hallbeck and Pedersen, 2008; Pedersen *et al*., 2008) or for hyporheic zones (Marxsen, 1996; Fischer and Pusch, 1999; Findlay and Sobczak, 2000; Marxsen, 2001). In most of these studies, nevertheless, spatial and temporal

resolution is generally poor with regard to providing full knowledge of the variability of microbial activities in space and time.

The present research was conducted in the aquifer system of Doñana (SW, Spain) with groundwater samples taken from 30 different piezometers over a two-year period. This paper represents the second step towards a detailed, ecological description of the microbial communities inhabiting this aquifer, and continues two previous studies in which these communities were described in terms of bacterial abundance, cell biomass and bacterial biomass (Velasco *et al*., 2009a; Velasco *et al*., 2009b). The aims of the present paper are: (1) to describe the activity of microbial communities by means of active microbial biomass, bacterial carbon production and bacterial growth rate at a large spatiotemporal scale, (2) to seek possible spatiotemporal variability in these functional variables, as well as the possible factors controlling them, and (3) to depict, from an ecological standpoint, a general view of microbial communities in this coastal, sandy aquifer system with data not only on activity, but also on bacterial abundance and biomass, considering not only the aquifer system *per se* but also the surrounding systems related thereto, such as shallow lakes.

MATERIALS AND METHODS

Site description

The Doñana Natural Protected Area is located in the southwest atlantic coast of the Iberian peninsula, covering an area of approximately 4000 km² and including the greater fluvial-littoral ecosystem of Doñana (Figure 5.1). This greater system encompasses four different types of ecosystems: marshes, aeolian mantles, coastal lines and estuaries. The importance of Doñana as a natural area lies not only in the diversity of its ecosystems: it is a major stepping-stone on the migration route of birds moving between Europe and Africa, it is home to the most endangered mammal in the world (the Iberian lynx, *Lynx pardina*), as well as many endemic, threatened or ecologically interesting species, and it contains perhaps the most significant wetland in Europe. In 1969, the Spanish government endowed Doñana with National Park status and its surroundings were declared as a Natural Park in 1989. Doñana was recognized as an International Biosphere Reserve in 1980, as a RAMSAR site in 1982 and as a UNESCO World Heritage Site in 1995 (Martín-López *et al*., 2007). The area presents a mediterranean climate with atlantic influence, generally classified as dry subhumid. Average annual temperature is 16.7 ºC. Average annual rainfall is 580 mm, but the variability is notable among and within years; most precipitation occurs during autumn and spring.

The most important source of freshwater in Doñana is the groundwater (Trick and Custodio, 2004). The aquifer of Doñana is a complex system that covers an area close to 3000 km² (Figure 5.1). Sedimentarily, the aquifer is comprised of detrital deposits from the Neogene period, covered by quaternary fluvio-marine and aeolian materials (Trick and Custodio, 2004). In general, these detrital sediments are not consolidated and overlap pliocene and miocene silts and marls constituting the impermeable lower boundary of the aquifer (Manzano and Custodio, 2007). From a

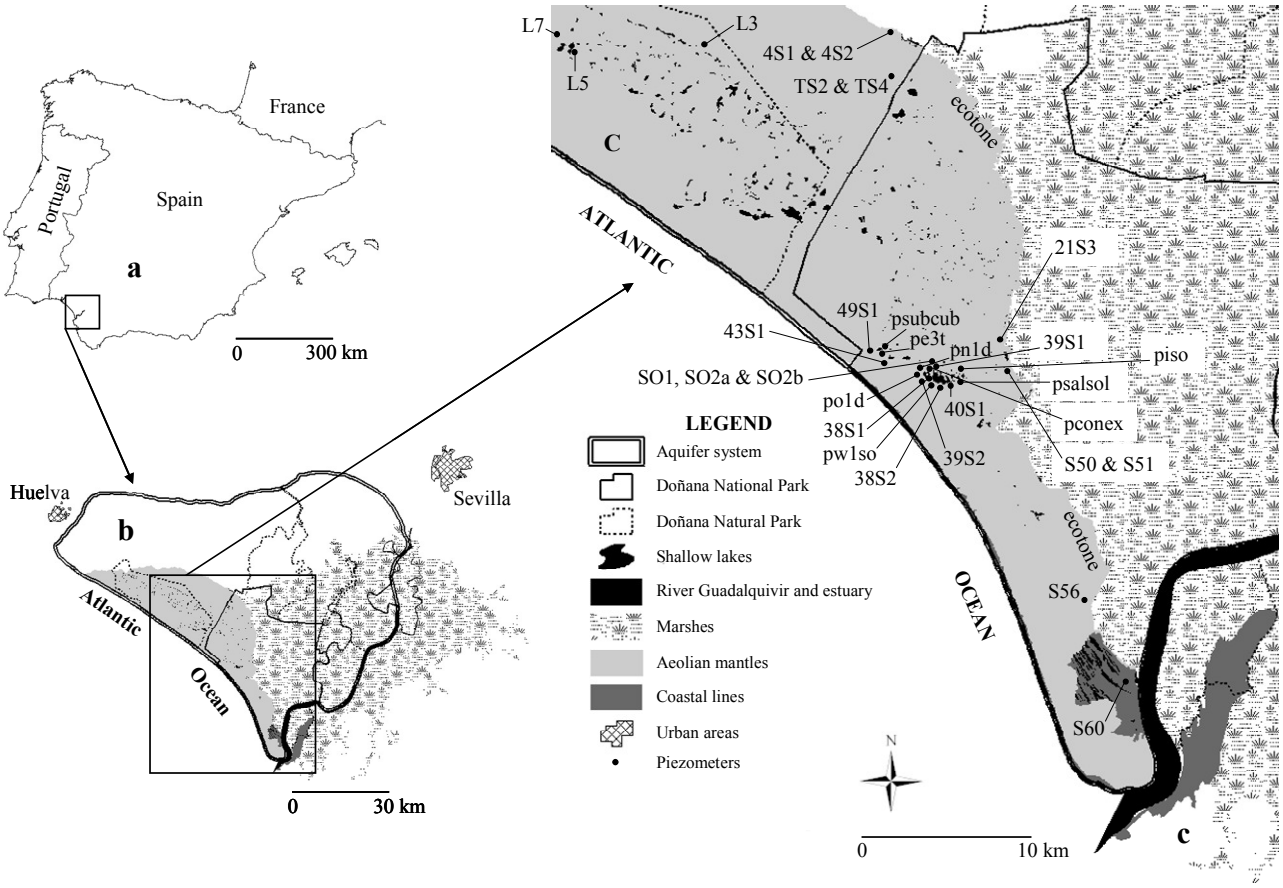


Figure 5.1 Geographical location of the greater fluvial-littoral ecosystem of Doñana on the southwest coast of the Iberian peninsula (a). Boundary of the Doñana aquifer system, limits of Doñana National and Natural Parks, main urban areas and limits of the four different types of ecosystems described in the natural protected area (b). Positions of the 30 piezometers studied over a two-year period and located over aeolian mantles (c).

hydrogeological point of view, the detrital deposits enable two main aquifer units to be distinguished. The aeolian mantles, which cover the western area of Doñana, encompass the phreatic unit, while the clayed materials, located in the eastern part of Doñana (mainly in the marshes), constitute the confined unit. Both units, however, are hydraulically connected and function as a whole (Trick and Custodio, 2004). In the phreatic unit, there are two different lithological domains (called upper and lower units) that allow the presence of two interconnected hydrodynamic units. The thick and fine-to-medium aeolian sands of the upper unit conform a relatively homogeneous, low permeability and unconfined upper aquifer that contains the water table and overlies a lower, less homogeneous, and semiconfined lower aquifer constituted by coarse sands and gravels. The hydraulic conductivity of the lower, thinner aquifer is higher than that of the upper aquifer. Between the upper and the lower units there is an intermediate layer of grey clays and fine-to-medium clayed sands containing iron oxide-minerals (Trick and Custodio, 2004). Groundwater recharge is produced by rainfall infiltration in the phreatic unit. Groundwater flows eastward from the aeolian mantles to the marshes. Natural discharge takes place to the ocean, to the rivers and ravines, to many small phreatic shallow lakes placed on top of the aeolian mantles (*e.g.*, Santa Olalla and Dulce shallow lakes), and through phreatic evapotranspiration. In the last few

decades, groundwater abstraction for agriculture and tourism has partially replaced natural discharge (Manzano and Custodio, 2007).

Table 5.1 Geographical and hydrogeological characteristics of the piezometers studied

Piezometers	Number of seasonal samples	Series	UTM X (29)	UTM Y (29)	Altitude (masl)[1]	Screen depth (mbls)[2]
psubcub	11	puam	721570	4096527	13.20	1.80-2.30
piso	5	puam	724598	4095905	5.50	2.00-2.25
pn1d	11	puam	724092	4095848	6.00	2.00-2.25
pw1so	9	puam	724408	4095430	6.50	2.00-2.25
pconex	11	puam	724186	4095769	6.30	2.20-2.40
pe3t	11	puam	721855	4096926	14.50	2.80-3.30
S51	6	S	727730	4097026	2.00	3.00-7.00
po1d	11	puam	723727	4095863	5.50	3.40-3.90
psalsol	11	puam	725178	4095599	6.00	3.50-3.75
21S3	4	SGOP	727969	4101313	4.00	5.40-8.20
40S1	11	SGOP	725165	4095562	6.30	6.40-9.40
L5	11	L	705087	4111029	68.00	8.00-10.00
4S1	7	SGOP	722485	4111933	4.38	8.00-10.00
L3	2	L	711081	4111414	43.00	8.00-10.00
L7	4	L	700031	4113890	69.00	8.00-10.00
38S2	9	SGOP	724490	4095425	6.00	8.10-11.00
39S2	5	SGOP	723848	4095698	5.70	8.50-11.50
TS4	4	T	719101	4112389	16.30	10.00-11.00
49S1	11	SGOP	721412	4096836	14.90	11.40-14.20
43S1	4	SGOP	722031	4096458	11.30	11.50-13.30
38S1	11	SGOP	724240	4095800	5.70	14.20-17.00
S60	3	S	734318	4080575	3.00	16.00-17.00
TS2	4	T	719101	4112389	16.30	18.00-19.00
39S1	11	SGOP	724107	4095773	5.80	18.00-21.70
SO2a	6	SO	724189	4096032	6.00	25.00-30.00
4S2	1	SGOP	722485	4111933	4.57	36.50-43.50
SO2b	6	SO	724189	4096032	6.00	44.00-46.00
S50	2	S	727730	4097026	3.00	52.00-60.00
S56	1	S	733010	4087500	2.00	74.00-80.00
SO1	7	SO	724188	4096038	6.00	67.00-72.00

[1]Metres above sea level
[2]Metres below land surface

Table 5.2 Lithological and hydrogeological characteristics of the piezometers studied

Piezometers	Screen lithology[1]	Shallow lakes[2]	Hydrogeological behaviour[3]	Hydrogeological unit[4]	Transmissivity ($m^2\ d^{-1}$)[5]	Permeability ($m\ d^{-1}$)[5]
psubcub	fs	T	outflowing	U		
piso	fms	SO-D	outflowing	U		
pn1d	fms	SO-D	outflowing	U		
pw1so	fs	SO-D	mainly outflowing	U		
pconex	fms	SO-D	inflowing	U		
pe3t	fms	T	outflowing	U		
S51	fs			U	1.13	0.28
po1d	fms	SO-D	inflowing	U		
psalsol	fms	SO-D	outflowing	U		
21S3	fs			U		
40S1	fms	SO-D	outflowing	U		
L5	fs	O		U	0.90	0.45
4S1	fms			U		
L3	fms			U	0.20	0.10
L7	cs			U	4.00	2.00
38S2	fs	SO-D	mainly outflowing	U		

Table 5.2 Continued

Piezometers	Screen lithology[3]	Shallow lakes[4]	Hydrogeological behaviour[5]	Hydrogeological unit[6]	Transmissivity ($m^2 d^{-1}$)[7]	Permeability ($m d^{-1}$)[7]
39S2	fms	SO-D	inflowing	U		
TS4	fms			U	3.00	3.00
49S1	fms	T	inflowing	U		
43S1	fms			U	0.13	0.05
38S1	fms	SO-D	inflowing	U		
S60	fs			U		
TS2	c			INT	0.50	0.50
39S1	fms	SO-D	inflowing	U		
SO2a	fms	SO-D	inflowing	U		
4S2	cfs			L		
SO2b	fms	SO-D	inflowing	U		
S50	gcs			L		
S56	gcs			L		
SO1	fms	SO-D	inflowing	U		

[1]Lithology of the screen section: fs, fine sands; fms, fine to medium sands; cs, coarse sands; c, clayed materials; cfs, clayed and fine sands; gcs, gravel and coarse sands
[2]Location of the well in the surrounding of shallow lakes: T, Toro; SO-D, Santa Olalla-Dulce; O, Oro
[3]Main hydrogeological flow direction for wells located in the vicinity of shallow lakes: *inflowing* means that water flows predominantly towards the pond while *outflowing* is towards the aquifer system
[4]Location of the wells in the hydrogeological units of the phreatic aquifer system: U, upper; L, lower; INT, intermediate
[5]Data from Trick (1998)

Sampling procedure: physical and chemical variables

Groundwater samples were collected seasonally, once per season in the same month over a two-year period (winter 2003 – winter 2005), from 30 piezometers located on the aeolian mantles, although some wells were not sampled in all seasons (Figure 5.1) (Table 5.1). SGOP-series wells were installed by the Guadalquivir river Basin Authority (CHG), puam-series piezometers were placed by the staff of the Universidad Autómoma de Madrid (UAM), and S-, L-, T- and SO-piezometers were installed by the Spanish Geological Survey (IGME). Wells only have one screened interval. The screen depth of the shallower well ranges from 1.80 to 2.30 meters below land surface (mbls), whereas the screen depth of the deeper well ranges from 74.00 to 80.00 mbls (Table 5.1). Groundwater samples were obtained with a submersible peristaltic pump (Uwitec, Mondsee, Austria) (Danielopol and Niederreiter, 1987) according to chemical *(*Dunlap *et al*., 1977) and microbiological standard procedures (Fredrickson *et al*., 1997). Groundwater was extracted from each piezometer until temperature (T), dissolved oxygen (DO), pH and electric conductivity (EC) stabilized (Fredrickson *et al*., 1997); samples were then taken. Physical variables (T, DO, pH and EC) were measured with a WTW 340i hanheld multiparameter device (WTW, Weilheim, Germany). Chemical variables (alkalinity, ammonium, nitrate, nitrite, soluble reactive phosphorus – SRP– and total phosphorus –TP–) were estimated by means of standard methods (APHA *et al*., 1987). Ferrous and ferric iron concentrations, as well as total iron, were determined by the ferrozine colorimetric method (Viollier *et al*., 2000).

Microbiological variables

Active microbial biomass (AMB), defined as the total amount of living or active cellular material, was determined by measuring the quantity of ATP, a method largely used due to its low detection limit, high level of precision and ease of performance (Karl, 1993; Eydal and Pedersen, 2007). We

collected groundwater samples to estimate AMB for each well and season in triplicate, storing them in autoclaved 50 mL glass bottles and placing them on ice during transport to the field laboratory. During the same sampling day, ATP determinations were performed via firefly luciferin-luciferase bioluminescence assay according to the instructions of a commercial kit (BioThema, Handen, Sweden). Briefly, groundwater samples (50 µL) were rapidly transferred to 10 mL sterile, plastic vials and amended with 100 µL of extractant reagent and 200 µL of luciferin-luciferase enzyme. Vials were immediately placed in a Berthold Junior LB09509 luminometer (Berthold Detection Systems, Pforzheim, Germany) and allowed to react during 10 seconds. Three replicated samples and two controls (made with bi-distilled and autoclaved water) were performed for each groundwater sample recovered. The relative light units (RLU), determined with the luminometer, were converted into ATP concentration units through a regression equation calculated with an ATP standard provided in the same commercial kit. ATP concentrations were transformed into carbon equivalents by means of the following relationship: 1 ng mL^{-1} of ATP per 250 ng mL^{-1} of bacterial carbon (Karl, 1993). AMB data are shown in ngC mL^{-1}. AMB analyses were performed during 2004 and 2005.

We collected groundwater samples to determine bacterial carbon production (BCP) in triplicate, for each well and season, storing them in autoclaved 100 mL glass bottles, leaving little or not headspace, and kept refrigerating them during transport to the field laboratory. BCP was measured through the incorporation of [^{3}H]leucine into cellular proteins over time (Kirchman, 1993), a method broadly applied in aquatic systems (Fischer and Pusch, 1999). Briefly, four 10 mL replicated samples and two formaldehyde-killed controls (4% v/v final concentration) were amended with L-[4,5-^{3}H]leucine (37MBq mol^{-1}; Amersham Biosciences, Chalfont St. Giles, UK) 30 nM final concentration, and incubated at 20 °C in the field laboratory; as a consequence, probably it would be better to talk about potential bacterial carbon production rates rather than bacterial carbon production rates because they were not estimated at *in situ* temperatures. The incorporation of [^{3}H]leucine in the four replicated samples was terminated with formaldehyde (4% v/v final concentration) after 2 hours. Following this, both samples and controls were frozen (-20 °C) until protein extraction procedure was carried out in the laboratory. The extraction procedure followed that of Kirchman (1993). Briefly, samples and controls were thawed and 50% TCA was added to obtain 5% TCA final concentration in order to initiate extraction. Samples and controls were immediately heated to 80°C for 20 minutes, cooled, and filtered through 0.45 µm nitrocellulose filters (Millipore, Billerica, MA, USA). Filters were then rinsed twice with cold 5% TCA and cold 80% ethanol, and placed in scintillation vials; when dry, 1 mL of ethylene glycol monomethyl ether was added to dissolve them. After this, 10 mL of scintillation cocktail were added to the scintillation vials, and radioactivity was measured in a LKB Wallack 1219 Rackbeta Liquid Scintillation Counter (LKB Instruments, Mount Waverley, Victoria, Australia). Quench correction was made by internal standardization. BCP was finally estimated according to the equation $BCP = LI \times 131.2 \times (\%\ Leu^{-1}) \times (C/protein) \times ID$, where LI is the leucine incorporation rate (mol L^{-1} h^{-1}), 131.2 is the formula weight of leucine, % Leu is the fraction of leucine in cellular proteins (0.073), C/protein is the ratio of cellular carbon to protein (0.86) and ID is the isotope

dilution (both external and internal) (Kirchman, 1993). ID was compensated by addition of sufficiently high concentrations of leucine in order to inhibit its *de novo* synthesis, a process that was therefore assumed not to have taken place in the present study. BCP data are shown in ngC mL^{-1} h^{-1}.

Bacterial growth rate (BGR), defined as the amount of time required to replace bacterial biomass, was calculated dividing bacterial biomass by bacterial carbon production (Kirchman, 2002). Bacterial biomass data were taken from Velasco Ayuso *et al*. (2009a; 2009b). BGR data are shown in days.

Other variables

Screen lithology data and location in the hydrogeological units of piezometers were obtained from the Spanish Geological Survey (IGME) databases. Hydrological behaviour of some piezometers in relation to local or regional groundwater flows was obtained from Sacks *et al*. (1992) and Coleto (2003). Transmissivity and permeability data in some piezometers were previously determined (Trick, 1998; Trick and Custodio, 2004) (Table 5.2). Monthly rainfall data were obtained with permission from a sampling station located in Doñana National Park (Spanish Meteorological Agency, AEMET).

Statistical analyses

A hierarchical, agglomerative cluster analysis (HACA) was performed with mean values of DO, EC, alkalinity, ammonium, nitrate, SRP, TP, ferrous iron and ferric iron groundwater as a classification method for grouping similar piezometers (Legendre and Legendre, 1998), although we only used data on wells sampled four times or more. Differences in the means of AMB, BCP and BGR among piezometers were tested in each season with a one-way analysis of variance (ANOVA). Temporal differences in the means of these microbiological variables were tested with a one-way analysis of variance (ANOVA) in wells sampled three times or more during the two-year study period. Tukey´s honestly significant difference test (HSD test) was employed, as an *a posteriori* method, to compare pairs of means after ANOVA testing. Normality of variables was examined using the Kolgomorov-Smirnov test, and variables were transformed when necessary and possible (Zar, 1998). Relationships among variables were explored using Pearson correlation coefficients or Spearman rank order correlations, depending upon whether the data set in question was parametric or non-parametric in nature. A principal component analysis (PCA), with seasons as the grouping factor, was performed as an ordination method in order to search for temporal patterns in microbiological variables. A discriminant analysis, with piezometers sampled four times or more during the study period as the grouping factor, was conducted in order to classify and discriminate among the centroids of all these boreholes, as well as to determine which groundwater physical, chemical and microbiological variables best accounted for differences among wells (Legendre and Legendre, 1998). A p value of 0.05 was set as the significant threshold for all statistical analyses. All statistical analyses were performed with Statistica 6.0 for Windows (Statsoft, Tulsa, OK, USA).

RESULTS

Physical and chemical variables

Eight different groups can be observed in the dendrogram after HACA. Some of these groups only contain one piezometer but others contain several wells (Figure 5.2). Piezometers of the group 2 (40S1, 39S2, 38S1 and 39S1) are representative of ascending groundwater flows and, in general, show low EC values and high Na and Cl contents in comparison with other wells. Piezometers piso, pn1d and pe3t show low EC values and high nitrate concentrations. Sampling points SO2a, SO2b and SO1 are deep piezometers which show groundwaters with low EC values but with moderate contents of carbonates. Wells psalsol and 38S2 are located in the surroundings of Santa Olalla shallow lake and show high EC values and organic matter contents; however, wells S51, 21S3 and L5 also show high organic matter contents but lower EC values than psalsol and 38S2. Piezometers pw1so and po1d characterize groundwaters with elevated contents of iron and organic matter (Figure 5.2).

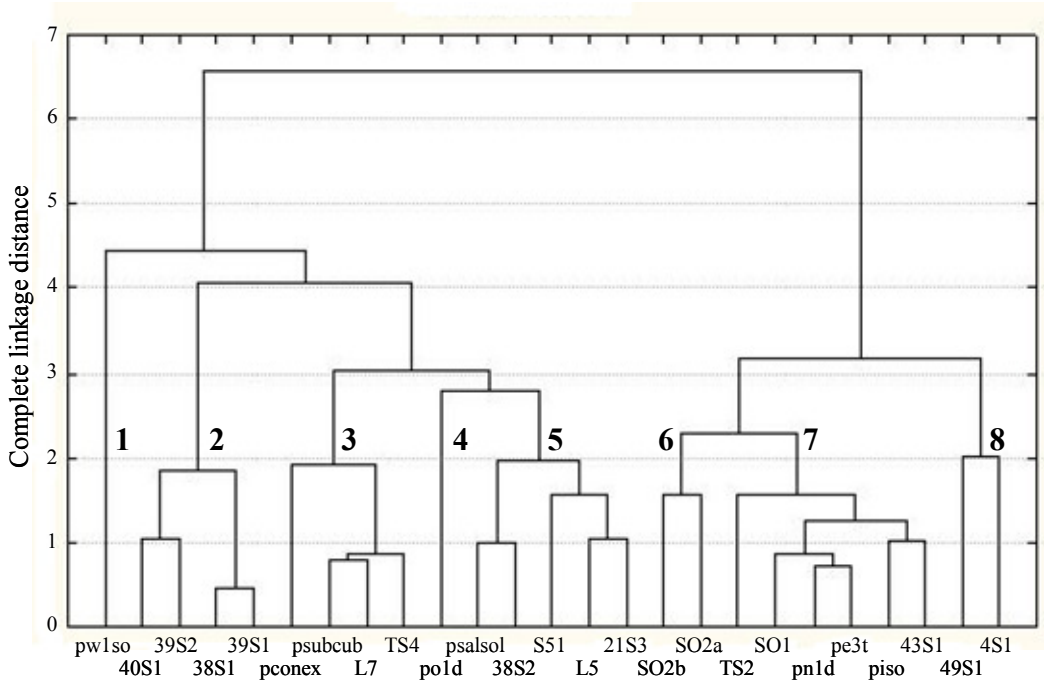


Figure 5.2 Hierarchical, cluster analysis of wells sampled four times or more during the study period (complete linkage clustering and Euclidean distances). Mean physical and chemical variables were used.

Microbiological variables

Active microbial biomass (AMB) showed a mean value of 2.54 ± 0.97 ngC mL^{-1} for all wells and seasons, ranging between $7.42 \times 10^{-3} \pm 2.20 \times 10^{-4}$ and 17.30 ± 3.71 ngC mL^{-1} (Figure 5.3). Significant seasonal differences were found in the means of AMB (ANOVA tests, $p \leq 0.036$). Although most boreholes showed significantly higher AMB values during summer or autumn than during spring 2004 (HSD tests, $p \leq 0.048$), some wells, such as pconex, S51, psalsol, L5 or 49S1, showed higher AMB values, significant in some cases, during spring than during summer or

autumn 2004 (HSD tests, $p \leq 0.032$). All wells showed significantly lower or statistically similar values in winter 2005 than during autumn 2004 (Figure 5.3). Boreholes 40S1, 39S2, 38S1 and 39S1 showed the highest AMB values, with no significant differences among them (HSD tests, $p \leq 0.027$), but with significant differences in relation to other wells in most seasons (HSD tests, $p \leq 0.036$). This variable positively correlated with DO ($r = 0.276$, $p = 0.016$, $n = 78$), SRP ($r = 0.240$, $p = 0.035$, $n = 78$), TP ($r = 0.269$, $p = 0.017$, $n = 78$), BCP ($r = 0.596$, $p = 0.000$, $n = 75$) and BGR ($r = 0.649$, $p = 0.000$, $n = 75$), and negatively with ferrous iron ($r = -0.475$, $p = 0.001$, $n = 59$). No correlations between AMB and rainfall were found in any of the piezometers. No correlations were found among AMB, screen depth, transmissivity and permeability in any season.

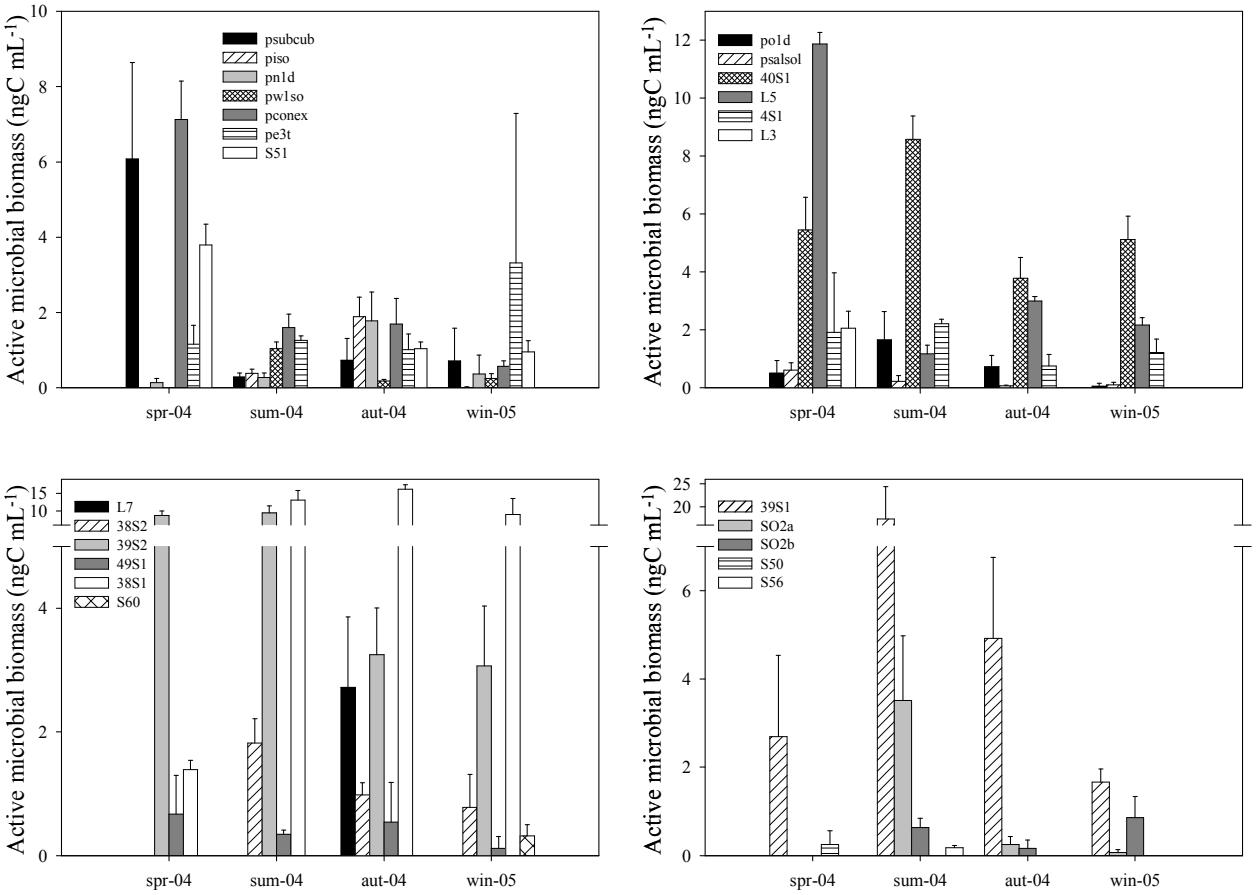


Figure 5.3 Seasonal changes in active microbial biomass (AMB) during the study period (win, winter; spr, spring; sum, summer; aut, autumn; solid bars indicate standard deviation).

Bacterial carbon production (BCP) ranged between $1.17 \times 10^{-3} \pm 2.36 \times 10^{-4}$ and 1.82 ± 0.21 ngC mL^{-1} h^{-1}, showing a mean value of 0.18 ± 0.72 ngC mL^{-1} h^{-1} in all wells and all seasons (Figure 5.4). All wells showed significant seasonal differences in the means of BCP (ANOVA tests, $p = 0.000$), except boreholes TS4 and TS2 (ANOVA tests, $p \geq 0.148$). In 2003, BCP was significantly higher during summer or autumn than during winter or spring in most piezometers (HSD tests, $p \leq 0.034$); however, wells such as pconex, psalsol or L5 showed significantly higher BCP during winter or spring than during summer or autumn 2003 (HSD tests; $p \leq 0.028$). In 2004, the temporal

pattern of BCP was very similar, with significantly higher values during summer or autumn than during winter or spring in most wells (HSD tests, $p \leq 0.038$). In winter 2005, most BCP rates decreased in relation to autumn 2004 (Figure 5.4). During 2003, wells 38S1 and 39S1 displayed maximum BCP rates in all seasons, with no statistical differences between them (HSD tests, $p \geq 0.328$) and with no significant differences in relation to BCP values measured in well L7 during winter and summer 2003 (HSD tests, $p \geq 0.259$) (Figure 5.4). During 2004, spatial patterns of BCP were more unclear, although wells 40S1 and 39S2 showed high BCP values, albeit without significant differences from other boreholes in some seasons (HSD tests, $p \geq 0.357$). BCP positively correlated with T ($r = 0.178$, $p = 0.017$, $n = 178$), SRP ($r = 0.359$, $p = 0.000$, $n = 178$), TP ($r = 0.376$, $p = 0.000$, $n = 178$), cell biomass ($r = 0.471$, $p = 0.021$, $n = 179$), bacterial biomass ($r = 0.165$, $p = 0.028$, $n = 179$) and AMB ($r = 0.596$, $p = 0.000$, $n = 75$). BCP positively correlated with rainfall from 2 months prior to sampling in wells S51, 21S3, 40S1, 39S2, 38S1, SO2b and SO1 ($r \geq 0.407$, $p \leq 0.037$, $n \geq 5$). BCP negatively correlated with screen depth in autumn 2003 ($r = -0.414$, $p = 0.032$, $n = 23$) and with permeability in summer 2003 ($r = -0.900$, $p = 0.037$, $n = 5$).

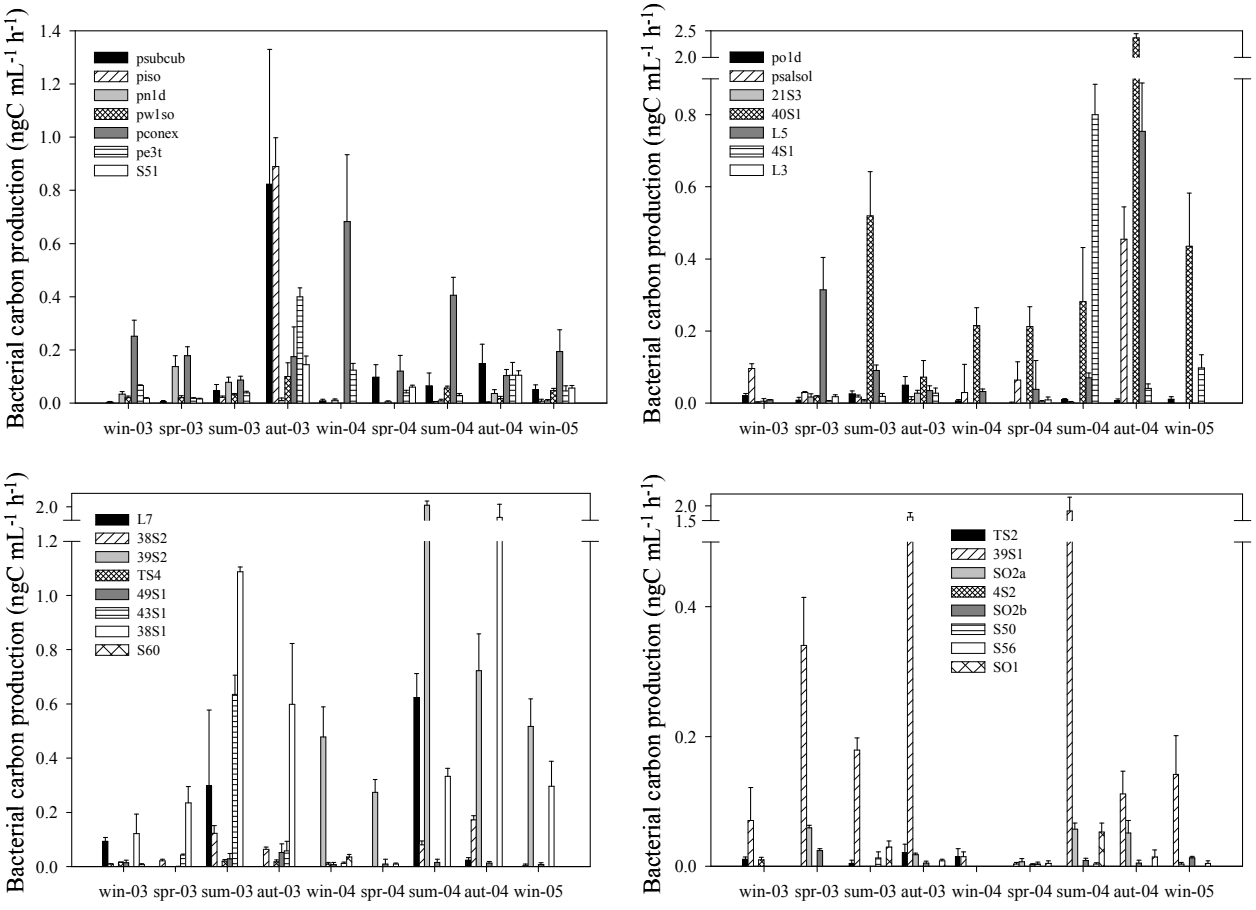


Figure 5.4 Seasonal changes in bacterial carbon production (BCP) (win, winter; spr, spring; sum, summer; aut, autumn; solid bars denote standard deviation).

Bacterial growth rate (BGR) ranged from 0.03 to 87.26 days and showed a mean value of 7.72 ± 8.58 days in all wells and seasons (Figure 5.5). The temporal pattern of this variable is erratic. During 2003, BGR values were usually lower during summer or autumn than during winter or

spring in most wells, except in wells psubcub and pconex (Figure 5.5). In contrast, during 2004 some wells showed peaks of BGR in summer (39S2, 39S1) or in autumn (psalsol, 40S1, L5, 38S1). During winter 2005, BGR values were normally higher than during autumn 2004 (Figure 5.5). BGR positively correlated with DO ($r = 0.177$, $p = 0.017$, $n = 179$), SRP ($r = 0.308$, $p = 0.004$, $n = 179$), TP ($r = 0.242$, $p = 0.001$, $n = 178$) and AMB ($r = 0.649$, $p = 0.000$, $n = 75$), while negatively with T ($r = -0.193$, $p = 0.040$, $n = 179$), bacterial abundance ($r = -0.550$, $p = 0.000$, $n = 179$) and cell biomass ($r = -0.292$, $p = 0.000$, $n = 179$). BGR found in wells S51, 21S3, 39S2, SO2b and SO1 positively correlated with rainfall 2 months prior to sampling ($r \geq 0.385$, $p \leq 0.009$, $n \geq 5$). Negative correlations were found between BGR and screen depth ($r = -0.451$, $p = 0.031$, $n = 23$) and between BGR and permeability ($r = -0.758$, $p = 0.028$, $n = 5$) during autumn 2003.

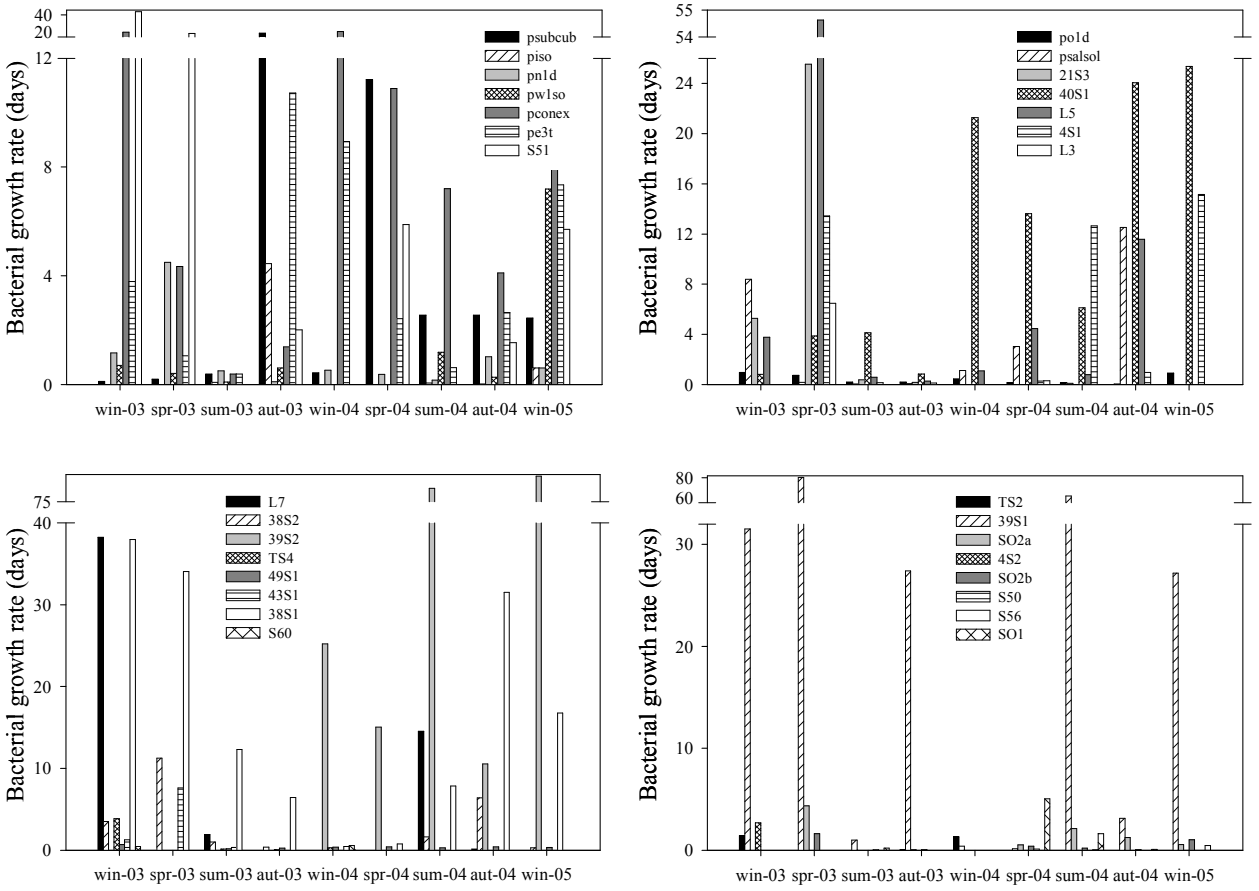


Figure 5.5 Seasonal changes in bacterial growth rate (BGR) during the study period (win, winter; spr, spring; sum, summer; aut, autumn).

The first three factors included in the principal component analysis (PCA), performed with five microbiological variables (bacterial abundance, cell biomass, AMB, BCP and BGR) and five physical and chemical groundwater variables (T, DO, SRP, TP and ferrous iron) of all wells sampled three times or more during the two-year study period, explained a total variance of 71.01% (Figure 5.6). Samples were classified into two major groups in relation to this principal component I: summer and autumn samples grouped on the right-hand side, while winter and spring samples grouped on the left. The first three canonical axes after discriminant analysis ($F_{240,1330} = 3.890$, $p =$

0.000) accounted for a 78.84% of the total variance (Figure 5.7). The first canonical axis explained 34.10% of total variance and showed an eigenvalue of 2.88. Bacterial abundance, DO and nitrate maximized differences and distances for the centroids of piezometers along the first canonical axis, whereas AMB, BCP, DO again, SRP and TP were the variables that allowed distribution of the centroids of the wells along the second canonical axis, which explained 30.52% of total variance and showed an eigenvalue of 2.58.

DISCUSSION

Physical and chemical variables

Hierarchical, cluster analyses have been defined as efficient methods for recognizing groups of groundwater samples that have comparatively similar physical and chemical characteristics (Güler *et al*., 2002). In this study, piezometers fed by nutrient-rich groundwater coming from the lower unit of the phreatic aquifer grouped together (40S1, 39S2, 38S1 and 39S1, group 2 in Figure 5.2). These piezometers represent regional, ascending groundwater flows and are characterized by high Na and Cl contents, mainly due to the proximity of the study area to the Atlantic ocean (Figure 5.1) (Coleto, 2003). Other piezometers, also fed by groundwater from the lower unit of the phreatic aquifer, grouped together in other clusters because they represent slightly different shallower (piso, pn1d, pe3t, group 7 in Figure 5.2) or deeper groundwaters (SO2a and SO2b, group 6 in Figure 5.2) than in the group 2 wells (Coleto, 2003). Shallow wells in group 7 are rich in nitrate. Deep wells SO2a and SO2b show high carbonate concentrations as a consequence of weathering processes in deep layers of the aquifer, very rich in marine shells (Coleto, 2003; Trick and Custodio, 2004). Two different subclusters appear in group 5 (Figure 5.2): one subgroup includes piezometers psalsol and 38S2, both characterized by salt water, mainly from shallow lakes and usually with high EC values resulting from evaporative processes in these surface aquatic systems, and another subgroup encompasses wells S51, 21S3 and L5, all fed by groundwater usually very rich in organic matter as a consequence of accumulation of detritic material, a very important process observed both in the ecotone (Figure 5.1) and in the interface between the sediment and water column of shallow lakes (Álvarez, 2002; Coleto, 2003). Some groups (1 and 4 in Figure 5.2) encompass a unique well (pw1so or po1d, Figure 5.2). Well pw1so contains mixed water between groundwater and water from Santa Olalla shallow lake (Coleto, 2003), while the groundwater of po1d is more similar to that of wells S51 or 38S2 than that of wells 38S1 or 39S1.

Microbiological variables

There are increasing evidences proving that not all cells in microbial communities are metabolically active (Haglund *et al*., 2002). Indeed, a proportion of active microbial biomass ranging from 0.02 to 6.36% of the total microbial biomass has been observed in the aquifer system of Doñana. Nevertheless, these proportions have to be taken with care because neither to calculate bacterial biomass nor to estimate active bacterial biomass fungi biomass, among other possible sources of microbial biomass, was kept into account. Any case, our results are similar to those found in shallow groundwater from sandy or gravelly deposits (Alfreider *et al*., 1997) or in the groundwaters

of crystalline aquifer systems of Sweden and Finland (Haveman and Pedersen, 2002). However, the reported fraction of active microbial biomass depends on the method used to estimate the proportion of active cells (Haglund *et al*., 2002) and, as a consequence, cross-comparisons among systems could produce false similarities. On the other hand, the active microbial biomass values (in terms of ngC mL^{-1}) found in the aquifer system of Doñana are more or less similar to those determined in shallow groundwaters (Eydal and Pedersen, 2007) or shallow subsurface sediments (Tietz *et al*., 2007).

From an ecological point of view, the production of carbon (or biomass) by heterotrophic bacteria is secondary production (Cole and Pace, 1995; Ducklow, 2000). Bacteria play a key role in organic carbon processing and influence many aspects of the chemistry and biology of aquifer systems (Goldscheider *et al*., 2006). Quantification of bacterial carbon production is therefore important for holistic studies in ecosystems (Fischer and Pusch, 1999). In this context, estimation of bacterial carbon production through the measurement of protein synthesis rates constitutes a widely used approach for gaining reliable information on the bulk activity of bacterial assemblages (Buesing and Gessner, 2003). Bacterial carbon production rates measured in the aquifer system of Doñana were similar to, although lower than, due to the seasonality of data, those observed for attached cells in shallow subsurface sediments (Tietz *et al*., 2007), shallow river sediments (Marxsen, 1996; Fischer and Pusch, 2001; Marxsen, 2001) or shallow lake sediments (Haglund *et al*., 2002), while were higher than that observed for free-living cells in shallow groundwaters (Alfreider *et al*., 1997) or hyporheic waters (Brugger *et al*., 2001). Although particle-associated microbial communities generally exhibit higher levels of activity than free-living microbial communities (Haglund *et al*., 2002), high rates of BCP shown by the free-living microbial communities of the aquifer system of Doñana are probably explained by the transmissivity of the aquifer (Trick and Custodio, 2004), which favours the movement of organic matter and nutrients throughout sands (Sophocleous, 2002). In fact, even moderate transmissivity values in aquifer systems induces pronounced hydrological shear stress and leads to resuspension of sediment-associated bacteria (Pronk *et al*., 2009), so the BCP determined in this work might probably reflect not only those rates shown by free-living microbial communities, but also these presented by particle-associated microbial communities, although it is necessary to bear in mind that data shown by Pronk *et al*. (2009) are taken from a karst aquifer system.

Growth rate is a fundamental property of all microorganisms and is especially informative of the activity of microbial populations that have the potential for increasing at exponential rates (Kirchman, 2002). Bacterial growth rates estimated in this paper reveal maximum turnover times of actively dividing cells, due to the fact that our counts of bacteria may also include inactive or dormant cells. Rates determined in this study are higher than those observed in other groundwaters (1.7-3.7 h) (Horn *et al*., 2004), although it should be pointed out that the rates reported by this article were calculated under laboratory conditions, and possible temporal and spatial variabilities shown by microbial communities in natural systems, including aquifer systems (Goldscheider *et al*., 2006), were therefore not taken into account. In river waters (33.2-71.5 h) (Fischer and Pusch, 2001), river sediments (12.1-44.3 h) (Fischer and Pusch, 2001) and shallow soil horizons (2.1-13.1

h) (Bååth, 1998), bacterial growth rates have been described as being faster than those found in the aquifer system of Doñana.

Factors controlling microbial activities in groundwater. Temporal and spatial patterns

Temperature is a variable that usually regulates microbial activities in ecosystems (Hoppe *et al*., 2002). Significant correlations between T and bacterial carbon production or bacterial growth rates have been described in several aquatic systems (Battin *et al*., 2004; Glud and Middelboe, 2004; Kritzberg *et al*., 2006; Alonso-Sáez *et al*., 2008). In this study, T positively correlated with BCP, as previously described (Apple *et al*., 2006), and negatively with BGR. Although a direct, positive temperature dependency of bacterial growth rate has previously been described (Kirchman and Rich, 1997; Apple *et al*., 2006), it is not unusual to find a slightly, negative relationship between these two variables, because the higher the T the more activity and carbon production the microbial communities showed, and the bacterial biomass/bacterial carbon production ratio tends to decrease, even if cell biomass also increases during warm periods or shows marginally higher values. In fact, in this aquifer system, cell biomass ranged over one order of magnitude (Velasco Ayuso *et al*., 2009a; Velasco *et al*., 2009b) whereas bacterial carbon production ranged over three orders of magnitude. Moreover, bacterial growth rates usually show a low positive dependence of T at high temperatures (Coveney and Wetzel, 1995), and in this aquifer system the mean temperatures was around 20 ºC.

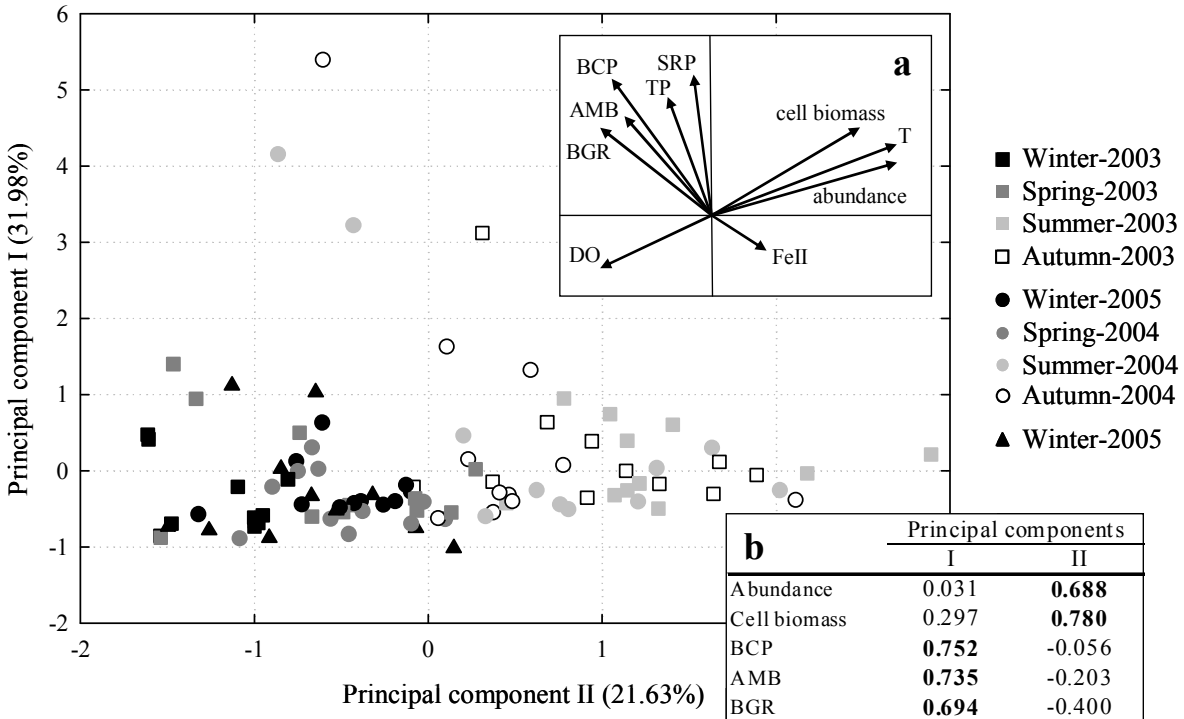
Unlike bacterial abundance or bacterial biomass, highly correlated with T in this aquifer system (Velasco Ayuso *et al*,. 2009a; Velasco *et al*., 2009b), BCP and BGR correlated only slightly with T, as it has been observed elsewhere (Sander and Kalff, 1993; Cole and Pace, 1995), which shows that other variables probably dominate and obscure a clear relationship among T, BCP and BGR. Among these other variables the quantity, and mainly the quality, of dissolved organic carbon has been identified as a relevant factor (Fischer and Pusch, 2001; Hoppe *et al*., 2002; Pulido-Villena and Reche, 2003; Kritzberg *et al*., 2006; Pedersen *et al*., 2008). Moreover, neither the proportion of active microbial biomass nor the active microbial biomass showed significant correlations with T, which highlights the idea that other factors appear to be exerting an influence on the activity of microbial communities in this aquifer system. In general, major factors controlling microbial activities usually include water availability and nutrient supply, but not always the temperature (Harris and Tibbles, 1997). However, although slight correlations among T, BCP and BGR were found, a temporal pattern in microbial activities, partially dependent on T, was observed during the two-year study period in the aquifer system of Doñana (Figure 5.6). Samples from autumn and summer grouped together, as did samples from winter and spring. Nevertheless, dispersion in summer and autumn samples was greater than in the winter and spring samples, probably reflecting a more important role played by T in controlling BCP and BGR when temperatures were lower (Sander and Kalff, 1993; Shiah and Ducklow, 1997). A very similar temporal pattern of microbial activities has been described in river sediments (Brugger *et al*., 2001) and shallow groundwaters (Alfreider *et al*., 1997).

Although a general decrease in AMB and BCP with depth has been described in river sediments (Marxsen, 1996), shallow sediments (Beloin *et al*., 1988) and groundwaters (Goldscheider *et al*., 2006; Eydal and Pedersen, 2007; Pedersen *et al*., 2008), only negative correlations between BCP or BGR and depth were found in the Doñana aquifer system during autumn 2003. Grain size is also considered an important factor controlling the activity of microbial communities in aquifer systems (Brockman and Murray, 1997; Musslewhite *et al*., 2003); in general terms, lithologies with the smallest pore throats, and thus low permeabilities, also present the lowest microbial activities (Sander and Kalff, 1993). However, the correlations between grain size and microbial activities were unclear throughout the two-year study period in the aquifer of Doñana, as it has previously been observed in other systems (Beloin *et al*., 1988). For example, well L7 has coarse sands in the screen region and did not show higher AMB values or BCP rates during any season; indeed, wells psubcub, L5 or 38S2, which have fine sands in the screen region, showed significantly higher BCP rates than L7 during autumn 2004 (HSD tests, $p \leq 0.036$) and statistically similar ones during summer 2003 and summer 2004 (HSD tests, $p \geq 0.365$). Moreover, negative correlations were found between BCP or BGR and permeability in this aquifer system only during summer and autumn 2003, although it should be kept in mind that only five values of permeability were used for these correlations (Table 5.2). In any case, no clear relationships among depth, permeability, AMB, BCP and BGR were found in this study and, consequently, the spatial patterns of microbial activities in the Doñana aquifer system cannot be adequately explained in terms of differences in depth or grain size. In fact, due to the different scales at which microbiological and geological processes exert their influences (millimetres to centimetres *vs* meters to kilometres), relationships between microbial activities and hydrogeological properties are difficult to describe or understand (Brockman and Murray, 1997; Fredrickson *et al*., 1997).

If neither depth nor grain size are key factors controlling the spatial patterns of microbial activities in the aquifer system of Doñana, we propose that groundwater flows, among other factors, play a relevant role in this sense. Spatial patterns of bacterial production and growth rate can be attributable to water and resource dynamics (Battin *et al*., 2004), and nutrient distribution in aquifer systems is mainly controlled by hydrogeological flows (Musslewhite *et al*., 2003; Zhou *et al*., 2004). Microorganisms generally show more activity at the ecotones, where gradients of electron donors, carbon sources and electron acceptors meet, than in homogeneous, mixed habitats (Pedersen *et al*., 2008). One important ecotone in an aquifer system, where spatiotemporal patterns in microbial activities can be found, is the contact between surface waters and groundwaters. In the surroundings of Santa Olalla and Dulce shallow lakes, two different water bodies that conform a unique one from a hydrological point of view (Coleto, 2003), well psalsol, the only one fed by water from Santa Olalla shallow lake all year round, showed significantly higher BCP rates than well po1d, fed by groundwater all year round, during winter and autumn 2003 (HSD test, $p \leq 0.023$), probably due to waters richer in nutrients coming from the shallow lake after a peak in primary production during the previous autumn (López-Archilla *et al*., 2004). However, during summer and autumn 2003, well po1d, among others, showed statistically higher BCP rates than psalsol (HSD tests, $p \leq 0.028$), probably because groundwater was richer in nutrients than water

coming from Santa Olalla shallow lake. During summer and autumn 2003, primary production was elevated in these shallow lakes (López-Archilla *et al*., 2004) and at the same time, nutrients and organic matter were effectively processed in the water column (Álvarez, 2002). Moreover, during summer and autumn 2003, well psalsol received less water from the shallow lake, because this water tends to flow predominantly in a vertical direction towards the aquifer system, thus recharging it (Sacks *et al*., 1992). During 2004, the situation was similar to what was found in 2003, except in autumn, when well psalsol showed significantly higher BCP rates than well po1d (HSD test, $p < 0.038$). This difference between years might be explained by differences in rainfall during the two hydrological cycles: 2003/2004 and 2004/2005. The second hydrological cycle was wetter than the first one and shallow lakes consequently showed higher levels during summer and autumn 2004 than during the same seasons in 2003 (Coleto, 2003). When shallow lakes have high water levels, they usually export water in a horizontal direction through outflowing wells, such as well psalsol. These horizontal hydrogeological flows act as a vector for nutrients and organic matter produced by primary producers in the surface waters. In a very similar study, Alfreider *et al*. (1997) described leucine incorporation rates in shallow sediments as being higher in outflowing areas than in inflowing ones during July, while they were lower during November and January. However, our data showed that the active microbial biomass was usually higher in inflowing wells than in psalsol during most part of 2004, including autumn 2004. It is therefore difficult to explain why these two activity variables present different temporal patterns, particularly if we take into account the positive correlation found between them. Nevertheless, this apparent lack of consensus between microbial activity variables does not necessarily represent a conflict, because it is very difficult to explain temporal and spatial patterns in microbial communities when large spatiotemporal scales are studied (Brockman and Murray, 1997; Musslewhite *et al*., 2003).

Other important ecotone in aquifer systems can be found at the contact between different lithological units, *e.g.* fine sands and coarse sands (Goldscheider *et al*., 2006), such as the contact found between the upper and the lower units of the phreatic aquifer of Doñana. From this contact, some hydrogeological flows ascend, carrying nutrients and organic matter. These flows might be the reason why some wells fed by deep groundwaters, such as 40S1, 39S2, 38S1 and 39S1, showed the highest AMB values and BCP rates during some seasons of both years (Figures 5.2 and 5.3). Curiously, these four piezometers grouped together in group 2 of the dendogram (Figure 5.2), showing that not only do they share similar microbiological characteristics, but also chemical and physical groundwater properties. In fact, it has been observed that high organic carbon concentrations appears to promote protein synthesis in balanced growth (Kirchman and Rich, 1997; Pulido-Villena and Reche, 2003) and that phosphate significantly stimulates AMB, BCP and BGR (Horn *et al*., 2004). Unfortunately, organic carbon concentrations were not determined in this study, but significant, positive relationships were found among SRP, TP, AMB, BCP and BGR. Moreover, other wells totally disconnected from shallow lakes, but also fed by deep, nutrient-rich groundwaters, such as L5 or L7, also showed high BCP rates or BGR values during some seasons (Figures 5.3 and 5.4). Therefore, results obtained in this aquifer system reveal that resource supply, among other factors, probably exerts an important influence on the magnitude of BCP. In general,



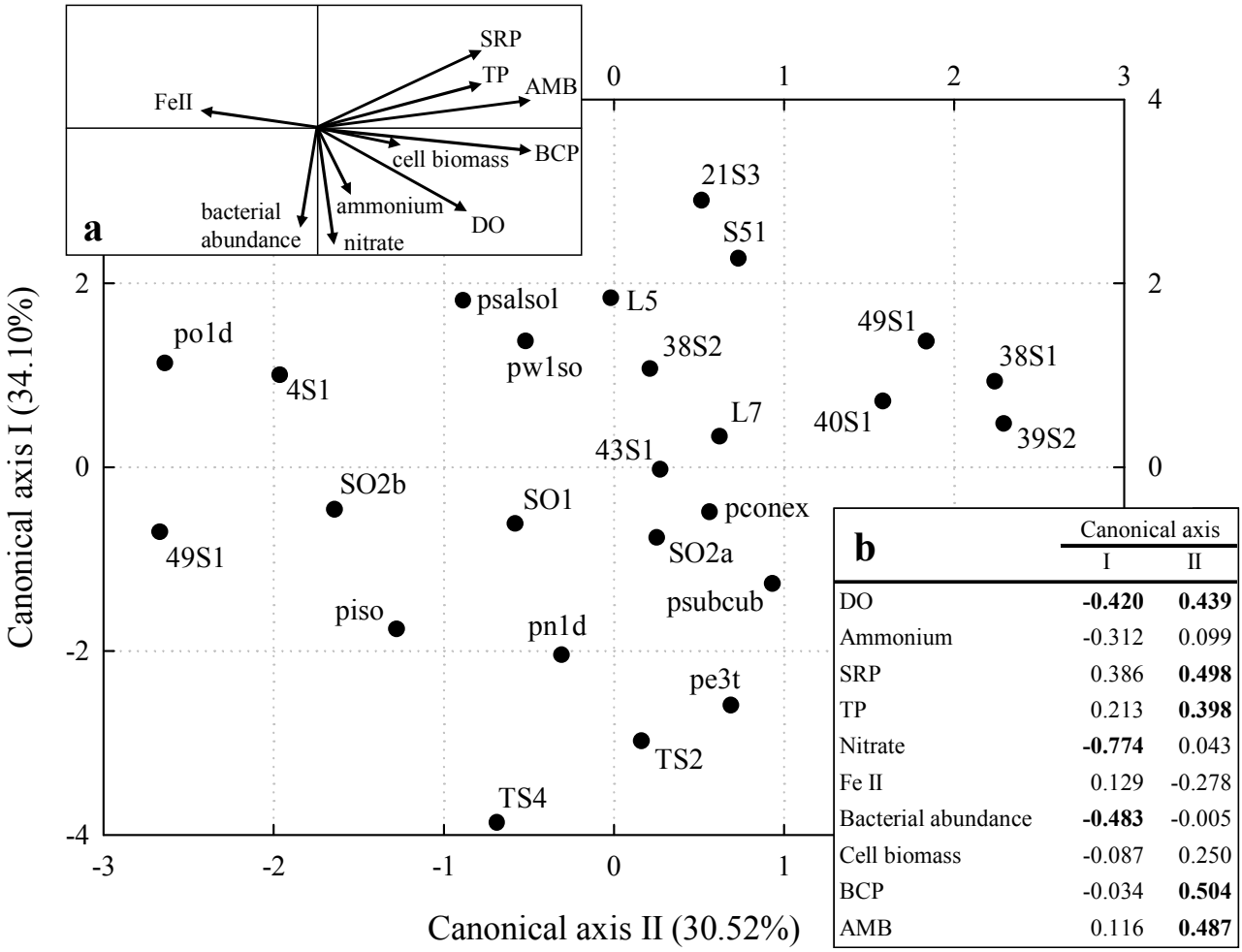
b

	Principal components	
	I	II
Abundance	0.031	**0.688**
Cell biomass	0.297	**0.780**
BCP	**0.752**	-0.056
AMB	**0.735**	-0.203
BGR	**0.694**	-0.400
T	0.254	**0.771**
DO	-0.266	**-0.663**
SRP	**0.878**	-0.100
TP	**0.850**	-0.035
FeII	-0.109	0.236

Figure 5.6 Scatter plot of principal component I *vs* principal component II showing the positions of the 175 observations for 25 different piezometers after principal component analysis (PCA). Seven descriptors plotted on the plane determined by the first two principal components (a). Factor loadings of these descriptors in both principal components (b) (boldface type indicates major factor loadings in each axis) (BCP, bacterial carbon production; AMB, active microbial biomass; BGR, bacterial growth rate; T, temperature; DO, dissolved oxygen; SRP, soluble reactive phosphorus; TP, total phosphorus).

microbial activities in aquatic systems usually show spatiotemporal patterns, partly as a result of temperature changes, but mainly due to variations in input of organic material and nutrients (Glud and Middelboe, 2004; Apple *et al*., 2006). Temperature likely regulates the magnitude of carbon metabolism at a relatively coarse temporal scale throughout the year, while finer spatial-scale variability at any given temperature is attributed to local environmental conditions, including nutrient concentrations and organic matter quality (Apple *et al*., 2006).

Positive correlations between rainfall 2 months prior to sampling and BCP (or BGR) in wells S51, 21S3, 40S1, 39S2, 38S1, SO2b and SO1 were found. All of these wells are curiously located in areas where important horizontal flows occur (lower unit, wells SO2b and SO1), strong ascending flows take place from deep areas of the aquifer system (deep wells located in the surroundings of shallow lakes, piezometers 40S1, 39S2 and 38S1, group 2 in Figure 5.2) or in areas where the phreatic aquifer discharges (ecotone in Figure 5.1, wells S51 and 21S3, group 5 in Figure 5.2). Consequently, these observations seem to suggest that regional hydrogeological flows play a relevant role in controlling the activity of microbial communities in some locations of this aquifer system, because they probably transport nutrients and organic matter. Indeed, activities of bacteria



b

	Canonical axis I	Canonical axis II
DO	**-0.420**	**0.439**
Ammonium	-0.312	0.099
SRP	0.386	**0.498**
TP	0.213	**0.398**
Nitrate	**-0.774**	0.043
Fe II	0.129	-0.278
Bacterial abundance	**-0.483**	-0.005
Cell biomass	-0.087	0.250
BCP	-0.034	**0.504**
AMB	0.116	**0.487**

Figure 5.7 Centroids of the 25 piezometers plotted along the first two canonical axes after discriminant analysis, showing both the contributions of the ten variables to the formation of the canonical axes (a) and the standardized canonical coefficients for the first two canonical axes (b) (boldface type denotes main variables on each canonical axis) (DO, dissolved oxygen; SRP, soluble reactive phosphorus; TP, total phosphorus; BCP, bacterial carbon production; AMB, active microbial biomass).

may be explained by factors that are general to bacteria in all habitats, *e.g.*, overall availability of nutrients and organic carbon, while habitat-specific factors, such as availability and structure of surfaces for attachment, are generally of subordinate importance (Haglund *et al*., 2002).

Finally, significant correlations found between both cell biomass and bacterial biomass with BCP can be explained if we take into account that, contrary to thymidine incorporation, leucine incorporation is more related to protein synthesis and cell growth than to cell duplication (Petit *et al*., 1999). Leucine incorporation reflects protein synthesis, and therefore measures an increase in bacterial biomass (Petit *et al*., 1999). In general, favourable environmental conditions might enhance bacterial protein and DNA synthesis, but they tend to favour the latter process in order to maximize reproduction over protein synthesis, and a strong correlation between T and bacterial abundance is more likely to be found than between T and BCP. Thus, it is not surprising that both active microbial biomass and bacterial carbon production are not correlated with bacterial abundance, mainly if the leucine incorporation is the method employed to measure bacterial carbon production (Wehr *et al*., 1999; Brugger *et al*., 2001; Gasol *et al*., 2002; Laybourn-Parry *et al*.,

2004). Generally speaking, in the Doñana aquifer system, the wells showing the highest bacterial abundances were not the same as those showing the highest BCP. High bacterial abundances were found in wells such as pw1so, po1d, L5, TS4 or TS2 (Velasco Ayuso *et al*., 2009a; Velasco *et al*., 2009b), while high BCP rates were found in piezometers such as S51, 40S1, 39S2, 38S1 and 39S1 (Figure 5.7). Moreover, canonical correlation analysis after discriminant analysis shows that wells that usually displayed the highest bacterial abundance values also exhibited high nitrate and ammonium concentrations, while piezometers with the highest BCP rates measured also showed relevant SRP and TP concentrations (Figure 5.7). Consequently, although significant correlations between microbiological and physicochemical variables in sedimentary aquifers are scarce (Martino *et al*., 1998; Musslewhite *et al*., 2003; Santoro *et al*., 2006), it seems that microbial activities in terms of active microbial biomass and bacterial carbon production are favoured by variables different from those favouring microbial communities in terms of bacterial abundance. Any case, it is very difficult to obtain simple and clear relationships between microbial and limnological variables in long time series and, when obtained, these relationships cannot be easily interpreted (Brockman and Murray, 1997; Mauck and Roberts, 2007).

CONCLUSIONS

The present study demonstrates the presence of active microbial communities in the aquifer system of Doñana. This is not surprising; microorganisms can appear and spread their activities, influencing the geochemical properties of their habitats, wherever enough space, nutrients and water are available for them to live (Ghiorse and Wilson, 1988). Moreover, this study establishes the first evidence of the key role played by the microbial communities in this aquifer system in the processing of organic matter. Previous studies have shown the important roles played by microbial communities in ecological processes associated with organic matter in other aquatic systems in Doñana, such as shallow lakes (Álvarez, 2002; Coleto, 2003; López-Archilla *et al*., 2004). Microbial communities can be expected to be equally significant in carbon processing in the Doñana aquifer system, especially if we take into account that both surface waters and groundwaters comprises a unique entity which functions as a whole (Manzano and Custodio, 2007). On the other hand, hydrogeological flows have been observed to be important in controlling microbial activities in the aquifer system of Doñana, mainly in those areas where these flows are relevant. Hydrogeological flows transport nutrients and organic matter and, as a consequence, microbial activities are enhanced in those areas with high concentrations of nutrients and organic matter. Thus, hydrogeology appears to exert a partial control over the spatial pattern of the microbial activities shown by microbial communities in this aquifer system. The temporal pattern of these microbial activities, although less clear than what has been found for bacterial abundance, cell biomass and bacterial biomass, is similar to that found in microbial communities present in surface aquatic systems (Álvarez, 2002). This similar temporal behaviour represents another evidence of the fact that groundwaters (at least shallow ones) and surface waters constitute a unique entity. Consequently, we propose that ecological studies in surface aquatic systems of Doñana, such as shallow lakes, should bear in mind not only the hypogenic wetlands, but also the aquifer system.

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CAPÍTULO 6. La función de las comunidades microbianas del sistema acuífero de Doñana II: actividades enzimáticas extracelulares

6. EXTRACELLULAR ENZYME ACTIVITIES IN A COASTAL, SANDY AQUIFER SYSTEM (DOÑANA, SW SPAIN)

Manuscrito enviado

ABSTRACT

A seasonal study of extracellular enzyme activities was conducted in the coastal, sandy aquifer system located in the greater fluvial-littoral ecosystem of Doñana (GED) (SW, Spain). ß-D-glucosidase, leucine aminopeptidase, alkaline phosphatase and phenol oxidase activities were determined over a two-year period in the groundwaters of 30 piezometers. Taking into account all enzymes, piezometers and seasons, extracellular enzyme activities (EEA) ranged over several orders of magnitude, from $1.01 \times 10^{-5} \pm 2.92 \times 10^{-6}$ to 1.37 ± 0.13 nmol (methylumbelliferyl, amido-4-methylcoumarin or dihydroxyphenylalanine) mL^{-1} h^{-1}. Neither temperature nor pH showed correlations with EEA. Other factors, such as organic matter and nutrient sources, seem to control the spatiotemporal patterns showed by EEA. Ratios of extracellular enzyme activities, which can be considered as measures of *microbial nutrient perception* varied among seasons as a consequence of several factors, including organic matter quality. Considering the hydrological flows that connect surface waters and groundwaters, the ecological role played by aquifer microbial communities in carbon and nutrient cycling processes, and the EEA measured in Doñana groundwater samples, we propose that the microbial communities inhabiting this aquifer system continue the decomposition process that begins in the sediments of the shallow lakes of Doñana, thus providing remineralized carbon and nutrients to higher trophic levels and playing a fundamental role in the cycling of materials within the GED.

6. ACTIVIDADES ENZIMÁTICAS EXTRACELULARES EN UN SISTEMA ACUÍFERO SEDIMENTARIO Y COSTERO (DOÑANA, SW ESPAÑA)

Manuscrito enviado

RESUMEN

En este estudio se presentan los resultados de las actividades enzimáticas extracelulares determinadas estacionalmente en el sistema acuífero costero y arenoso localizado en el gran ecosistema fluviolitoral de Doñana (GED) (suroeste de España). Las actividades de la β-D-glucosidasa, la leucina aminopeptidasa, la fosfatasa alcalina y la fenol oxidasa fueron determinadas a lo largo de un período de estudio de dos años en las aguas subterráneas de 30 piezómetros diferentes. Teniendo en cuenta todas las enzimas, piezómetros y estaciones, las actividades enzimáticas extracelulares (AEE) oscilaron entre $1.01 \times 10^{-5} \pm 2.92 \times 10^{-6}$ y 1.37 ± 0.13 nmol (metilumbeliferona, amino-4-metilcumarina y dihidroxifenilalanina) mL^{-1} h^{-1}. Ni la temperatura ni el pH mostraron correlaciones con las AEE, lo que sugiere que otras variables controlaron esas AEE. Otros factores, como por ejemplo diferentes fuentes de materia orgánica y de nutrientes, parecen ejercer una influencia importante en los patrones espaciotemporales mostrados por las AEE. Las proporciones entre las AEE, consideradas como una medida de la *percepción microbiana de los nutrientes*, variaron a lo largo de las diferentes estaciones como consecuencia de la acción conjunta de varias variables, entre las que la calidad de la materia orgánica parece tener un papel relevante. Teniendo en cuenta los flujos hidrológicos que conectan las aguas superficiales y las subterráneas, el papel ecológico que juegan las comunidades microbianas de los sistemas acuíferos en los procesos de reciclado de materia orgánica y nutrientes, así como las AEE medidas en las aguas subterráneas de Doñana, este estudio propone que las comunidades microbianas que habitan este sistema acuífero continúan los procesos de descomposición que comienzan en los sedimentos de las lagunas de Doñana, aportan nutrientes y compuestos remineralizados de carbono a niveles tróficos mayores y juegan, por tanto, un papel ecológico fundamental en los ciclos biogeoquímicos de materiales en el GED.

INTRODUCTION

Three decades ago, most microbiologists believed that active microbial communities were limited to topsoil and rhizosphere environments or to at most the upper 100 m of the Earth's crust (Griebler and Lueders, 2009). However, it is now recognized that the biosphere extends thousands of meters below the land surface (Kieft *et al.*, 1998), and that microbial communities are widespread in the Earth's subsurface systems. These communities appear to be metabolically and phylogenetically more diverse than previously thought (Goldscheider *et al.*, 2006; Griebler and Lueders, 2009). Therefore, aquifer systems are not semi-deserts occupied by exotic lineages but are dynamic systems comparable in complexity to surface systems (Humphreys, 2009). Subsurface microbial communities play fundamental ecological roles at several scales in organic matter processing and energy fluxes. Studies carried out in marine waters (Alonso-Sáez *et al.*, 2008; Bhaskar and Bhosle, 2008) suggest that deep microbial communities are major consumers of organic matter and that they provide two essential ecosystem services: a food source for higher trophic levels and the regeneration of nutrients through the remineralization of organic detritus (Harbott and Grace, 2005) while meeting their energy and carbon demands.

Groundwater ecosystems are generally devoid of photosynthesis and lack inputs of fresh, easily available organic carbon (Griebler and Lueders, 2009). As a consequence, groundwater generally tends to have a lower concentration of organic matter, which is primarily composed of larger polymeric molecules, than surface aquatic systems (Lehman and O'Connell, 2002). Dissolved organic matter is the largest pool of organic material in the water column in all aquatic systems (Münster and de Haan, 1998), including groundwater. This pool of organic matter can be visualized as a size continuum of polymers, macromolecular aggregates and colloids. Most of the components of this pool have high molecular weights; consequently, they cannot be used directly by microorganisms. Extracellular enzymes degrade these polymers, supporting the metabolism of bacteria and other osmotrophs that assimilate carbon and nutrients (Sinsabaugh and Follstad Shah, 2010a). The hydrolysis of polymers mediated by extracellular enzymes is considered to be the rate-limiting step in the utilization of high molecular weight dissolved organic matter (Chróst, 1992) because there is a fundamental limitation on the size of molecules that can be transported across biological membranes to fuel microbial metabolism (Pusch *et al.*, 1998). Thus, these enzymes are an important link between organic matter and microorganisms in aquatic environments (Chróst, 1992) and play a fundamental role in the recycling of inorganic and organic carbon and nutrients in aquatic systems (Miettinen *et al.*, 1996), including groundwaters.

Spatiotemporal patterns of extracellular enzyme activities (EEA) have been widely studied in lakes, rivers and marine waters. However, few studies have examined extracellular enzyme activities in aquifer systems in general or in groundwaters in particular (Miettinen *et al.*, 1996; Hendel and Marxsen, 1997; Kieft *et al.*, 1998; Hendel *et al.*, 2001; Lehman and O'Connell, 2002; McCarty *et al.*, 2007; Cooney and Simon, 2009; Kolehmainen *et al.*, 2009). The present study was conducted in the aquifer system of Doñana (SW, Spain). Groundwater samples were taken from 30 wells over a two-year period. To our knowledge, this is the first study to evaluate EEA in

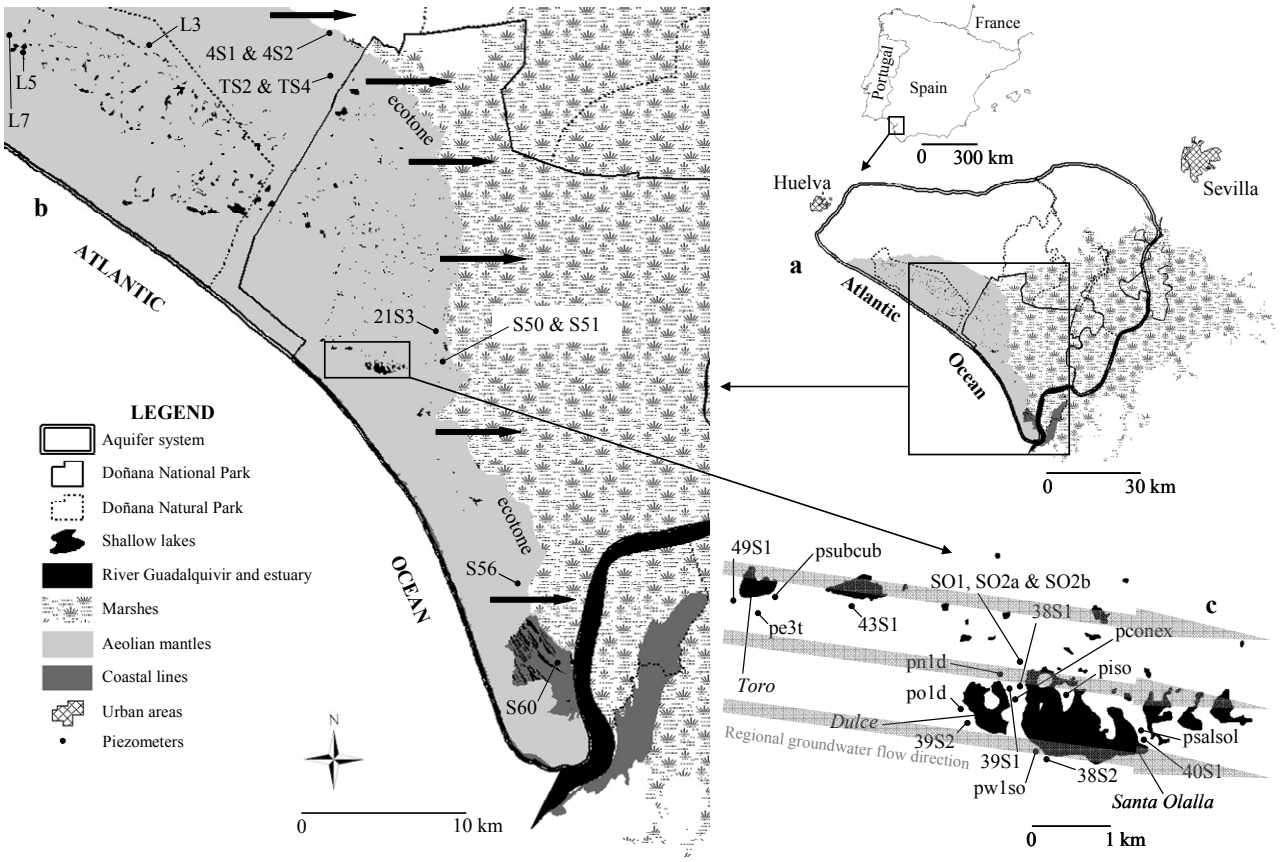

Figure 6.1 Geographical location of the greater fluvial-littoral ecosystem of Doñana (GED) in southwestern Spain, showing the limits of the aquifer system, the Doñana National Park, the Doñana Natural Park and the Guadalquivir river (a). Positions of the 30 piezometers located over aeolian mantles that were studied over a two-year period (b and c). Arrows indicate the general direction of regional groundwater flows at a global scale (b) or at a local scale within the region of several shallow lakes denoted in *italics* (c).

groundwaters in southern Europe. This study also contributes to a detailed ecological description of the microbial communities inhabiting the groundwaters of this aquifer system, building upon three previous studies that have defined these communities in terms of structural (bacterial abundance, cell biomass, bacterial biomass) and functional variables (active microbial biomass –AMB–, bacterial carbon production –BCP–, bacterial growth rate –BGR–) (Velasco Ayuso *et al.*, 2009a; Velasco Ayuso *et al.*, 2009b; Velasco Ayuso *et al.*, 2010). In this study, we aimed (1) to assess the magnitude and behaviour of EEA exhibited by microbial communities in the Doñana aquifer system; (2) to identify the variables that control the spatiotemporal patterns of EEA; and (3) to examine the ecological relationships between shallow lakes and groundwaters, two of the compartments that make up the greater fluvial-littoral ecosystem of Doñana (GED).

STUDY AREA

The greater fluvial-littoral ecosystem of Doñana (GED) is located along the lower stretch of the Guadalquivir river in southwestern Spain and is associated with the flat, dynamic coastal areas of the littoral zone and with the mouth of the river itself (Figure 6.1). Constituting perhaps the most representative example of a beach-dune-wetland-estuary system in southern Europe, the GED encompasses four types of ecological systems: marshes, aeolian mantles, coastal lines and estuaries

(Custodio *et al.*, 2009). In 1969, the Spanish government endowed Doñana with National Park status. Its surroundings were declared a Natural Park in 1989. Doñana was recognized as an International Biosphere Reserve in 1980, as a RAMSAR site in 1982 and as a UNESCO World Heritage Site in 1995 (Custodio *et al.*, 2009). The area presents a sub-humid mediterranean climate with atlantic influence and is generally classified as dry sub-humid. The average annual temperature is 16.7 ºC. The average annual rainfall is 580 mm, but the precipitation is notably variable within and among years; most precipitation occurs during the autumn and spring.

The diversity of aquatic ecosystems is perhaps the most relevant factor in the designation of Doñana as a nature reserve. Groundwater is the most important source of freshwater in Doñana (Trick and Custodio, 2004). The aquifer system of Doñana is a complex system that covers an area of approximately 2600 km² and is probably the largest coastal aquifer in Spain (Custodio *et al.*, 2009) (Figure 6.1). The lithological and hydrogeological features of this aquifer system have been described elsewhere (Manzano and Custodio, 2007; Custodio *et al*., 2009).

Table 6.1 Geographical and hydrogeological features of the selected piezometers

Piezometers	Series	Number of seasonal samples	UTM X (29)	UTM Y (29)	Altitude (masl)[1]	Screen depth (mbls)[2]	Hydrogeological unit[3]	Hydrogeological behaviour
psubcub	puam	11	721570	4096527	13.20	1.80-2.30	U	inflowing
piso	puam	5	724598	4095905	5.50	2.00-2.25	U	inflowing
pn1d	puam	11	724092	4095848	6.00	2.00-2.25	U	inflowing
pw1so	puam	9	724408	4095430	6.50	2.00-2.25	U	mainly outflowing
pconex	puam	11	724186	4095769	6.30	2.20-2.40	U	inflowing
pe3t	puam	11	721855	4096926	14.50	2.80-3.30	U	
S51	S	6	727730	4097026	2.00	3.00-7.00	U	
po1d	puam	11	723727	4095863	5.50	3.40-3.90	U	inflowing
psalsol	puam	11	725178	4095599	6.00	3.50-3.75	U	outflowing
21S3	SGOP	4	727969	4101313	4.00	5.40-8.20	U	
40S1	SGOP	11	725165	4095562	6.30	6.40-9.40	U	inflowing
L5	L	11	705087	4111029	68.00	8.00-10.00	U	
4S1	SGOP	7	722485	4111933	4.38	8.00-10.00	U	
L3	L	2	711081	4111414	43.00	8.00-10.00	U	
L7	L	4	700031	4113890	69.00	8.00-10.00	U	
38S2	SGOP	9	724490	4095425	6.00	8.10-11.00	U	mainly outflowing
39S2	SGOP	5	723848	4095698	5.70	8.50-11.50	U	inflowing
TS4	T	4	719101	4112389	16.30	10.00-11.00	U	
49S1	SGOP	11	721412	4096836	14.90	11.40-14.20	U	inflowing
43S1	SGOP	4	722031	4096458	11.30	11.50-13.30	U	inflowing
38S1	SGOP	11	724240	4095800	5.70	14.20-17.00	U	inflowing
S60	S	3	734318	4080575	3.00	16.00-17.00	U	
TS2	T	4	719101	4112389	16.30	18.00-19.00	INT	
39S1	SGOP	11	724107	4095773	5.80	18.00-21.70	U	inflowing
SO2a	SO	6	724189	4096032	6.00	25.00-30.00	U	inflowing
4S2	SGOP	1	722485	4111933	4.57	36.50-43.50	L	
SO2b	SO	6	724189	4096032	6.00	44.00-46.00	U	inflowing
S50	S	2	727730	4097026	3.00	52.00-60.00	L	
S56	S	1	733010	4087500	2.00	74.00-80.00	L	
SO1	SO	7	724188	4096038	6.00	67.00-72.00	U	inflowing

[1]Meters above sea level
[2]Meters below land surface
[3]Location of the wells in the hydrogeological units (U, upper; L, lower; INT, intermediate)

MATERIALS AND METHODS

Sampling procedure: physical and chemical groundwater variables

Groundwater samples were collected seasonally (once per season) over a two-year period from 30 piezometers located on the aeolian mantles within an area encompassing approximately 100 km² (Figure 6.1). However, not all wells were sampled during all seasons (Table 6.1). Groundwater samples were obtained using a submersible peristaltic pump (Uwitec, Mondsee, Austria) (Danielopol and Niederreiter, 1987), following standard chemical (Dunlap *et al.*, 1977) and microbiological procedures (Fredrickson and Phelps, 1997). Groundwater was extracted from each piezometer until temperature (T), dissolved oxygen (DO), pH and electric conductivity (EC) measurements stabilized; samples were then taken. Physical variables (T, DO, pH and EC) were measured with a WTW 340i handheld multi-parameter device (WTW, Weilheim, Germany). Chemical variables (alkalinity, ammonium, nitrate, nitrite, soluble reactive phosphorus –SRP– and total phosphorus –TP–) were estimated using standard methods (APHA *et al.*, 1987). Ferrous (FeII) and ferric iron (FeIII) concentrations, as well as total iron (TFe), were determined by the ferrozine colorimetric method (Viollier *et al.*, 2000).

Microbiological variables

Groundwater samples for estimating EEA were collected from each piezometer in triplicate, stored in 50 mL polyurethane bottles (pre-washed in 5% HCl and distilled water) and frozen until experiments began (within the next ten days). In the laboratory, groundwater samples were thawed and then assayed for the activity of four different enzymes involved in the degradation of cellulose (ß-D-glucosidase, GLU, EC 3.2.1.21) or polyphenolic compounds (phenol oxidase, PHO, EC 1.10.3.2) to assimilate C and in the acquisition of N (leucine aminopeptidase, LAP, EC 3.4.11.1) or P (alkaline phosphatase, APE, EC 3.1.3.1). Due to problems with sample preservation, only enzyme data from the last five seasons are considered here, except in the case of PHO. Artificial fluorogenic or chromogenic substrates were used to determine EEA. All enzyme assays were conducted at saturating substrate concentrations to facilitate comparison of total enzyme activities among samples. Therefore, the measured values should be considered potential activities rather than estimates of *in situ* activities. GLU, LAP and APE activities were determined in triplicate from winter 2004 to winter 2005 using fluorogenic model substrates, as specified by Sinsabaugh *et al.* (1997) and Sinsabaugh and Foreman (2001). Substrates for GLU, LAP and APE were 4-methylumbelliferyl-ß-D-glucopyranoside (MUF-ß-D-glucopyranoside), L-leucine-7-amido-4-methylcoumarin (Leu-AMC) and 4-methylumbelliferyl-phosphate (MUF-phosphate), respectively (Sigma-Aldrich, Saint Louis, Missouri, USA). Substrate solutions (1 mM final concentration) were prepared in autoclaved 5 mM sodium bicarbonate buffer (pH 8.0) and stored at 4 ºC in the dark. To improve the solubility of MUF and AMC compounds, 2 mL of hydroxymethyl ether were added to each substrate solution. GLU, LAP and APE activities were determined in black 96-well microplates. Fluorescence readings for each microplate were recorded at 1 h intervals during 24 h using a Perkin-Elmer LS 50B luminescence spectrometer (Perkin-Elmer, Waltham, Massachusetts, USA) under optimized conditions of a 365 nm wavelength slit width excitation filter and a 450 nm

wavelength slit width emission filter. The microplates were kept in the dark at 20 ºC and shaken continuously to reduce cell adhesion to the well walls. The final substrate concentration in each well for the enzyme assays was 0.2 mM. Activities were expressed as the rate of accumulation of MUF or AMC equivalents, as appropriate, in units of nmol mL^{-1} h^{-1}. Quenching was estimated by comparing the fluorescence of methylumbelliferone or 7-amino-4-methyl coumarin standards mixed with groundwater samples to the fluorescence of 50 µL of standard solution mixed with 200 µL of a 5 mM sodium bicarbonate solution. PHO activities were determined in triplicate from winter 2003 to winter 2005 using L-3,4-dihydroxyphenylalanine (DOPA) (Sigma-Aldrich, Saint Louis, Missouri, USA) as a chromogenic model substrate, according to the methods of Sinsabaugh and Findlay (1995) and Sinsabaugh (2010). Fresh DOPA was always used. PHO assays were carried out at 20 ºC in 5 mL polypropylene test tubes in the dark. The tubes were shaken continuously. After one hour of incubation, the tubes were centrifuged, and the supernatants were transferred immediately to cuvettes to measure their absorptions at 460 nm using a Varian Cari 1C spectrophotometer (Varian Medical Systems, Palo Alto, California, USA). PHO activity values are presented in units of nmol DOPA mL^{-1} h^{-1}.

Other variables

Hydrological data for some piezometers in relation to local or regional groundwater flows was obtained from and Sacks *et al.* (1992) and Coleto (2003). The locations of the piezometers in the hydrogeological units were obtained from the Spanish Geological Survey (IGME) databases (Table 6.1).

Extracellular enzymes stoichiometry and the MARCIE model

EEA can be considered as a product of microbial heterotrophic production that mediates resource consumption and nutrient flows, representing an intersection of metabolic and stoichiometric ecological theories. In the context of these theories, the extracellular enzyme ratios GLU/LAP and GLU/APE are limited by the stoichiometric ratios $TER_{C:N}/B_{C:N}$ and $TER_{C:P}/B_{C:P}$ and by the metabolic ratios A_N/GE and A_P/GE (Sinsabaugh *et al.*, 2009; Sinsabaugh *et al.*, 2010), where $TER_{C:N}$ and $TER_{C:P}$ are the threshold elemental ratios of C:N and C:P, $B_{C:N}$ and $B_{C:P}$ are the elemental C:N and C:P ratios of microbial biomass, A_N and A_P are the assimilation efficiencies for N and P, and GE is microbial growth efficiency with respect to C. By using normalized EEA to bacterial carbon production rates (BCP), it is possible to compare the relationships between EEA and heterotrophic metabolism in microbial communities (Sinsabaugh *et al.*, 2010). According to Sinsabaugh *et al.* (2009) and Sinsabaugh *et al.* (2010), the ratios of normalized EEA to BCP rates can be linked to both microbial metabolism and environmental resource availability in the Doñana groundwater samples through the following relationships:

$$[1]\ GLU/LAP \sim (TER_{C:N}/B_{C:N}) = (A_N/GE)$$
$$[2]\ GLU/LAP \sim (TER_{C:P}/B_{C:P}) = (A_P/GE)$$

Liebig´s classical law of the minimum is now being replaced by a more general approximation that considers microbial communities to continually allocate energy to acquire multiple resources, including C, N and P (Sinsabaugh *et al.*, 2010). In fact, the MARCIE model (model for *Microbial Allocation of Resources among Community Indicator Enzymes*) proposes that BCP is maximized through an optimal resource allocation strategy that trades off C, N and P-acquiring enzyme activities as environmental carbon and nutrient availabilities change (Sinsabaugh *et al.*, 1994; Sinsabaugh *et al.*, 1997; Foreman *et al.*, 1998). The MARCIE model predicts that BCP rates are directly related to C flow, represented by enzymes involved in C acquisition (E_C, in this study by GLU), and are not directly related to the sum of the activities of all enzymes. However, BCP is constrained by the need to acquire N and P, represented by enzymes involved in the acquisition of N (E_N, in this study by LAP) and P (E_P, in this study by APE). Consequently, E_C can also be expressed as a fraction of total extracellular enzyme production (E_T, the sum of E_C, E_N and E_P), whose value is dependent on N (E_N/E_C) and P (E_P/E_C) limitation (see Sinsabaugh *et al.*, 1994 for the complete derivation):

$$[3]\ BCP = k_C E_T/(1+E_N/E_C+E_P/E_C)$$

To evaluate this model, it is necessary to test whether BCP rates are proportional to E_C activity and whether C, N and P acquisition are linked through an optimum resource allocation strategy. In other words, it is necessary to determine whether BCP is directly related to the ratios E_C/E_N and E_C/E_P, which represent the availability of nitrogen and phosphorus, respectively, as perceived by microbial communities (Álvarez, 2002).

Statistical analyses

Differences in the means of each of the four EEA among different wells were tested for each sampling campaign by using a one-way ANOVA test. Seasonal differences in the means of each of the four EEA were analysed in each piezometer by means of a one-way ANOVA test, but only if three or more seasons were sampled. Differences among the means of the four different EEA in each piezometer and each season were also tested by a one-way ANOVA test. Several analyses of variance tests were carried out because the data matrix was not regular, *i.e.*, not all piezometers were sampled all seasons (Table 6.1). In order to compare pairs of means after ANOVA testing, an *a posteriori* Tukey's HSD test was performed. Relationships among normalized to BCP C, N and P-acquiring enzyme activities were determined in each season by calculating regression coefficients after natural logarithm (ln) transformation of data. Seasonal and total differences between normalized to BCP lnGLU *vs* lnLAP and lnGLU *vs* lnAPE regressions were tested by using ANCOVA tests. Relationships among variables were explored using Pearson product moment correlations or Spearman rank order correlation, depending on whether the dataset in question was parametric or non parametric in nature. A principal component analysis (PCA), with wells in which all the EEA were estimated as the grouping variable, was carried out as a multivariate ordination method to seek for spatial and temporal patterns (Legendre and Legendre, 1998). Normality was examined by means of the Shapiro-Wilk test, and variables were transformed when necessary and when possible (Zar, 1998). A p value of 0.05 was set as the significant threshold for all statistical

analyses. All statistical analyses were performed using the SPSS 17.0 software for Windows (Statistical Product and Services Solutions, Inc., Chicago, Illinois, USA).

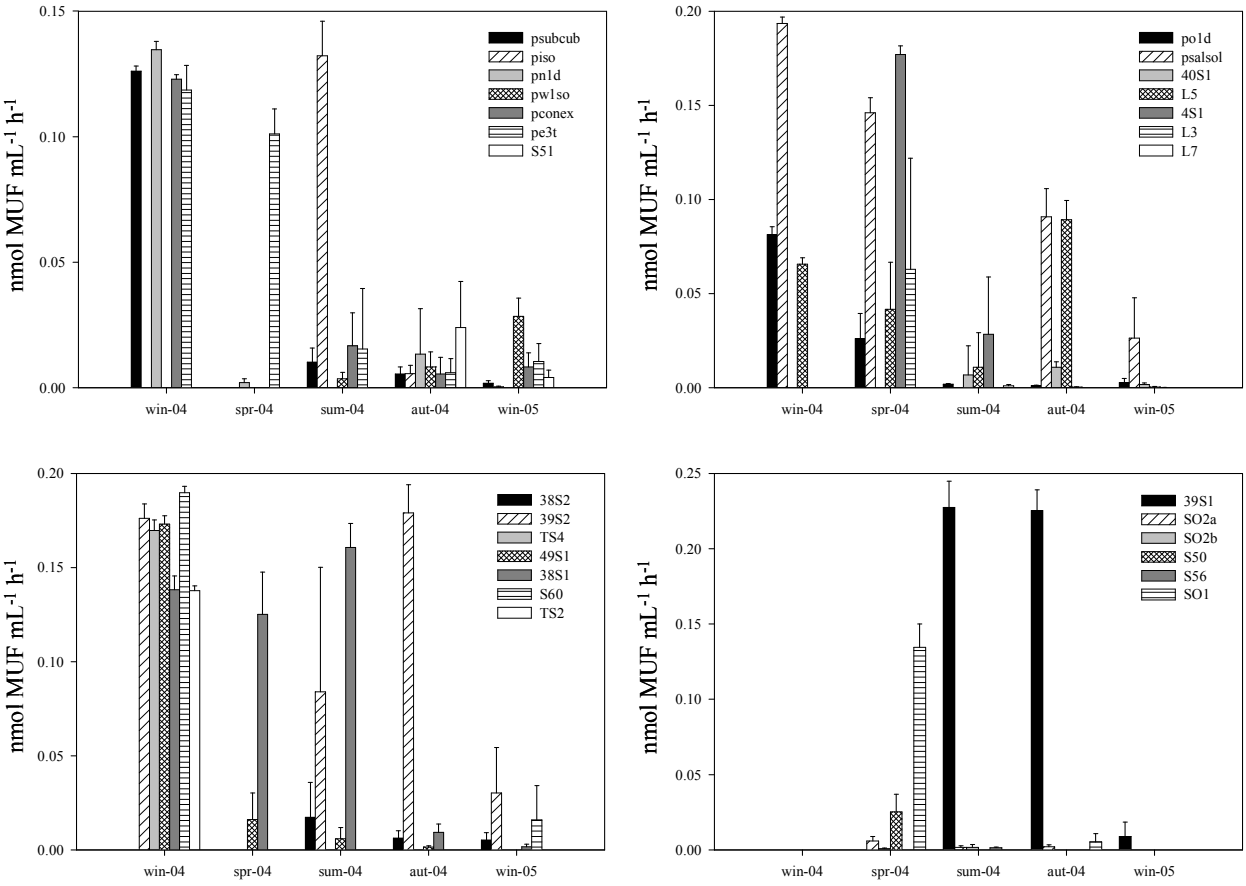

Figure 6.2 Seasonal changes in GLU (ß-D-glucosidase) activities. Means (columns) and standard deviations (bars) were calculated from triplicate samples from each piezometer (winter 2004, win-04; spring 2004, spr-04; summer 2004, sum-04; autumn 2004, aut-04; winter 2005, win-05).

RESULTS

The piezometer 4S1 showed the lowest GLU activity value, which occurred during winter 2005 ($1.35 \times 10^{-4} \pm 7.66 \times 10^{-5}$ nmol MUF mL^{-1} h^{-1}), whereas 39S1 showed the highest GLU activity value, which occurred during summer 2004 (0.22 ± 0.01 nmol MUF mL^{-1} h^{-1}) (Figure 6.2; in this figure, piezometers are grouped by screen depth). GLU activities were positively correlated with TP ($r = 0.312$, $p = 0.005$, $n = 79$), nitrate ($r = 0.216$, $p = 0.033$, $n = 71$), BCP rate ($r = 0.425$, $p = 0.029$, $n = 76$), AMB ($r = 0.259$, $p = 0.042$, $n = 62$), LAP activity ($r = 0.730$, $p = 0.000$, $n = 60$) and APE activity ($r = 0.545$, $p = 0.000$, $n = 74$). LAP activities varied between $1.01 \times 10^{-5} \pm 2.92 \times 10^{-6}$ and 0.19 ± 0.04 nmol AMC mL^{-1} h^{-1}, showing their minimum values in the piezometer piso during autumn 2004 and their maximum values in the piezometer 39S2 in summer 2004 (Figure 6.3; in this figure, piezometers are grouped by screen depth). LAP activities were positively correlated with TP ($r = 0.345$, $p = 0.005$, $n = 66$), AMB ($r = 0.457$, $p = 0.001$, $n = 52$), BGR ($r = 0.328$, $p = 0.007$, $n = 66$), APE activity ($r = 0.632$, $p = 0.000$, $n = 63$), GLU/APE ratio ($r = 0.447$, $p = 0.000$, $n = 57$) and

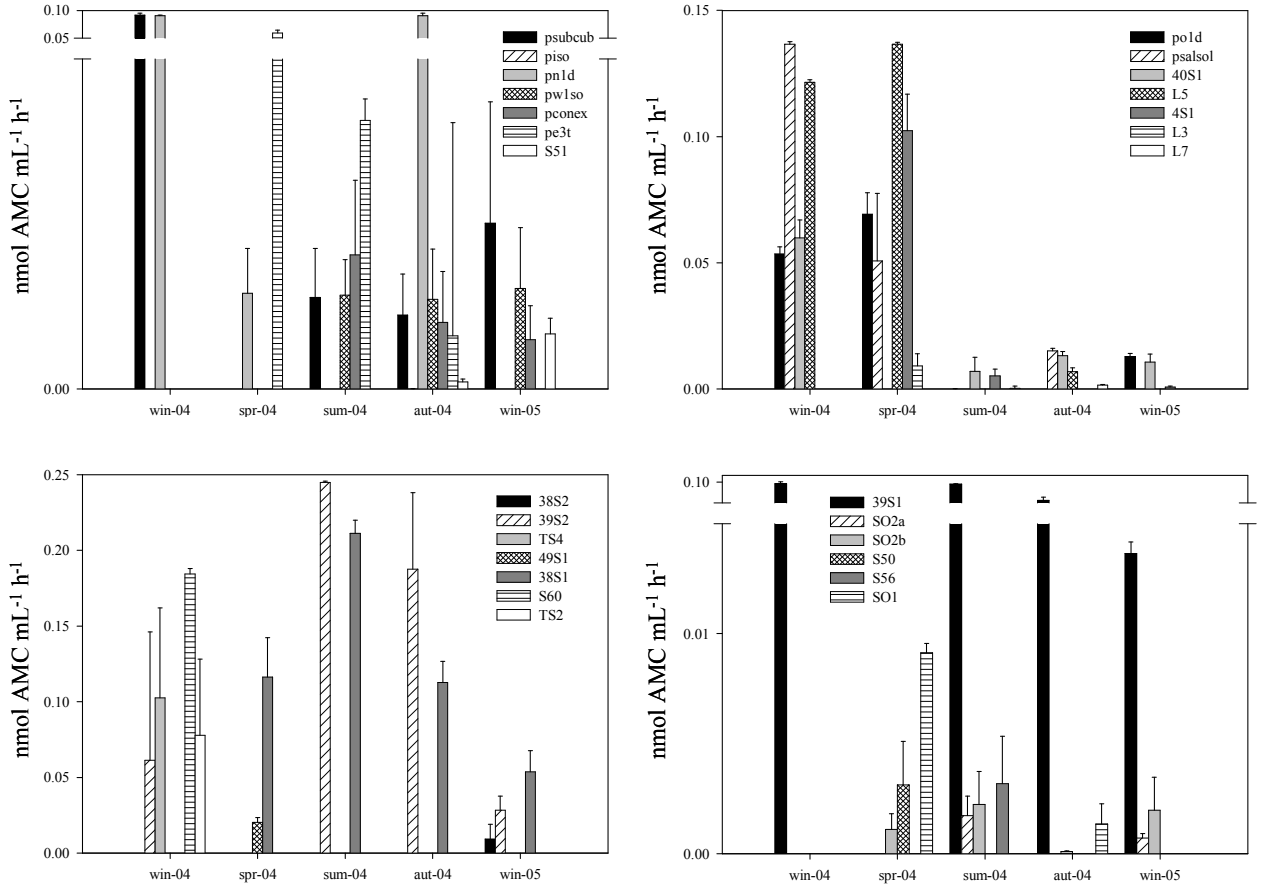

Figure 6.3 Seasonal changes in LAP (leucine aminopeptidase) activities. Means (columns) and standard deviations (bars) were calculated from triplicate samples from each piezometer (winter 2004, win-04; spring 2004, spr-04; summer 2004, sum-04; autumn 2004, aut-04; winter 2005, win-05).

GLU/PHO ratio (r = 0.589, p = 0.000, n = 60). APE activities were lowest during spring 2004 in the piezometer pn1d and highest in the piezometer S60 during winter 2004, ranging from $1.02 \times 10^{-3} \pm 9.71 \times 10^{-4}$ to $0.39 \pm 6.30 \times 10^{-2}$ nmol MUF mL^{-1} h^{-1} (Figure 6.4; in this figure, piezometers are grouped by screen depth). APE activity values were positively correlated with alkalinity (r = 0.290, p = 0.007, n = 86), TP (r = 0.286, p = 0.048, n = 86), BCP (r = 0.454, p = 0.020, n = 83), AMB (r = 0.351, p = 0.003, n = 70), BGR (r = 0.225, p = 0.041, n = 83) and GLU/PHO ratio (r = 0.379, p = 0.001, n = 74). PHO activity values ranged from $1.81 \times 10^{-2} \pm 1.01 \times 10^{-3}$ (piezometer psubcub, summer 2004) to 1.37 ± 0.13 nmol DOPA mL^{-1} h^{-1} (piezometer psalsol, autumn 2004) (Figure 6.5; in this figure, piezometers are grouped by screen depth). PHO activities were positively correlated with T (r = 0.218, p = 0.004, n = 170), EC (r = 0.510, p = 0.000, n = 170), TP (r = 0.342, p = 0.000, n = 169), FeII (r = 0.618, p = 0.000, n = 113), FeIII (r = 0.419, p = 0.000, n = 150), TFe (r = 0.638, p = 0.000, n = 150), alkalinity (r = 0.526, p = 0.000, n = 149), bacterial abundance (r = 0.201, p = 0.009, n = 170), cell biomass (r = 0.313, p = 0.000, n = 170) and bacterial biomass (r = 0.260, p = 0.001, n = 160) and negatively correlated with nitrate (r = -0.431, p = 0.000, n = 162) and DO (r = -0.491, p = 0.000, n = 170).

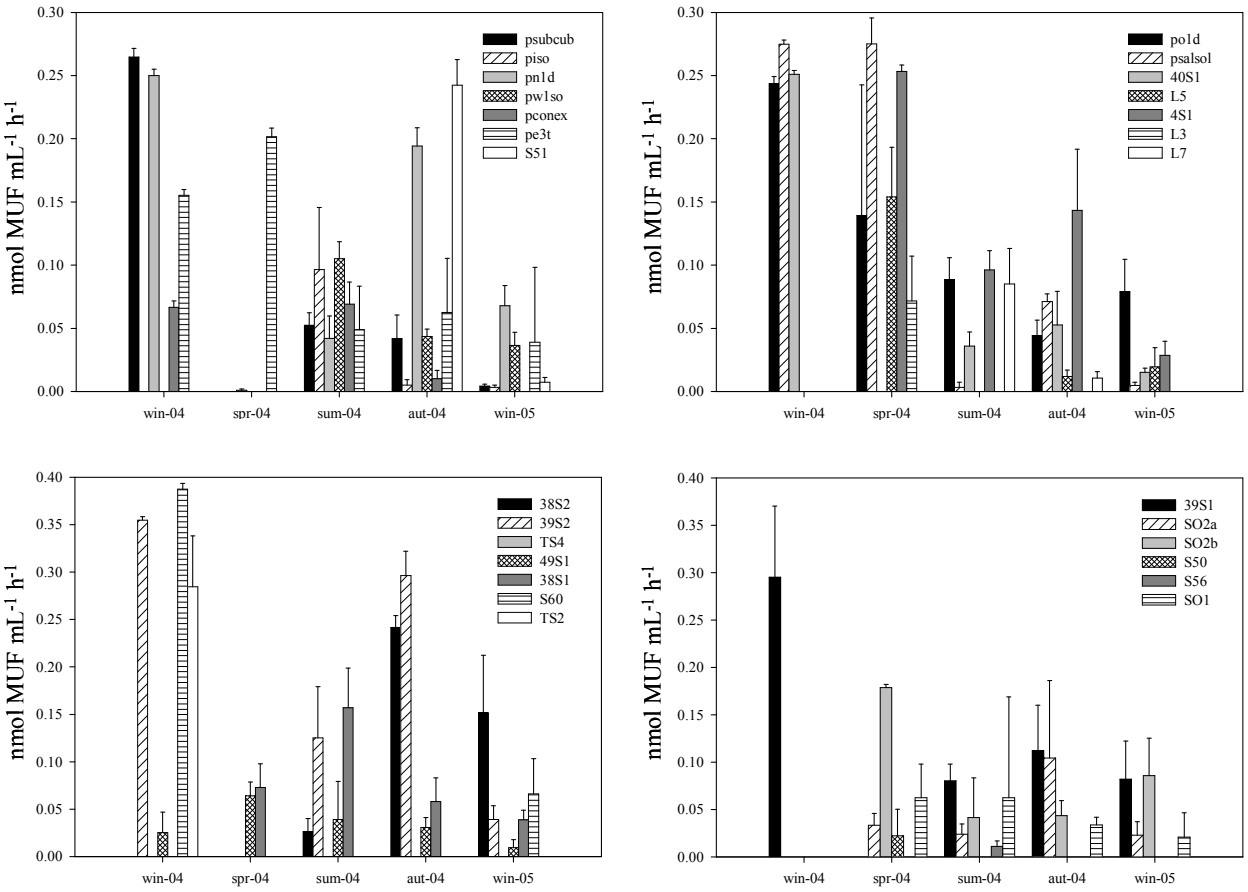

Figure 6.4 Seasonal changes in APE (alkaline phosphatase) activities. Means (columns) and standard deviations (bars) were calculated from triplicate samples from each piezometer (winter 2004, win-04; spring 2004, spr-04; summer 2004, sum-04; autumn 2004, aut-04; winter 2005, win-05).

Both seasonal and total results for relative nutrient availabilities (or *microbial perception of nutrients*) as measured by relationships among normalized to BCP lnGLU, lnLAP, lnAPE and lnPHO activities are shown in Table 6.2. Linear regressions were always significant for the relationships lnGLU *vs* lnLAP and lnGLU *vs* lnAPE, except during autumn 2004. The linear regression for the relationship lnGLU *vs* lnPHO, an index used to estimate the relative abundance of recalcitrant carbon (Sinsabaugh and Follstad Shah, 2010b), was significant only during winter 2005. The slopes of the regressions lnGLU *vs* lnLAP and lnGLU *vs* lnAPE were significantly greater during summer 2004 than during the other seasons (ANCOVA, $p \leq 0.003$). However, no significant differences in the slopes of the regressions lnGLU *vs* lnLAP and lnGLU *vs* lnAPE were found in any season (ANCOVA, $p \geq 0.458$), nor when all the data were considered together (ANCOVA, $p \geq 0.897$). Normalized to BCP lnGLU *vs* lnLAP ratio was positively correlated with bacterial abundance ($r = 0.290$, $p = 0.024$, $n = 60$), cell biomass ($r = 0.282$, $p = 0.029$, $n = 60$) and bacterial biomass ($r = 0.318$, $p = 0.013$, $n = 60$). Normalized to BCP lnGLU *vs* lnAPE ratio was positively correlated with DO ($r = 0.250$, $p = 0.035$, $n = 71$), nitrate ($r = 0.249$, $p = 0.039$, $n = 63$) and LAP activity ($r = 0.271$, $p = 0.042$, $n = 57$) and negatively correlated with pH ($r = -0.276$, $p = 0.021$, $n = 71$), EC ($r = -0.259$, $p = 0.029$, $n = 71$), TP ($r = -0.286$, $p = 0.048$, $n = 71$), FeIII ($r = -0.316$, $p = 0.007$, $n = 71$) and TFe ($r = -0.298$, $p = 0.012$, $n = 71$). Normalized to BCP lnGLU *vs* lnPHO ratio was positively correlated with DO ($r = 0.398$, $p = 0.000$, $n = 76$), nitrate ($r = 0.472$, $p = 0.000$, $n =$

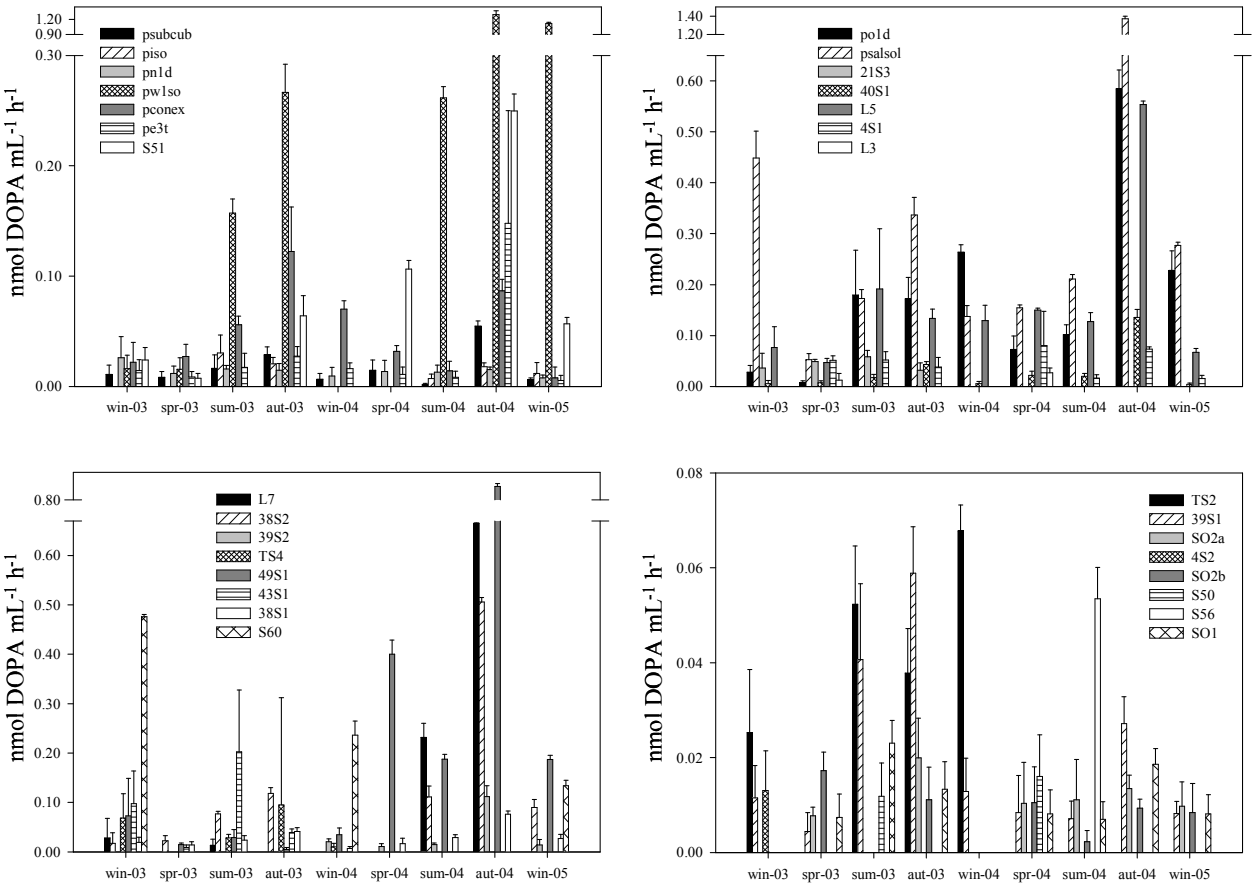

Figure 6.5 Seasonal changes in PHO (phenol oxidase) activities. Means (columns) and standard deviations (bars) were calculated from triplicate samples from each piezometer (winter 2003, win-03; spring 2003, spr-03; summer 2003, sum-03; autumn 2003, aut-03; winter 2004, win-04; spring 2004, spr-04; summer 2004, sum-04; autumn 2004, aut-04; winter 2005, win-05).

78), AMB (r = 0.404, p = 0.002, n = 59), LAP activity (r = 0.419, p = 0.001, n = 60) and APE activity (r = 0.326, p = 0.023, n = 71) and negatively correlated with T (r = -0.239, p = 0.038, n = 76), EC (r = -0.457, p = 0.000, n = 76), FeII (r = -0.413, p = 0.003, n = 50), FeIII (r = -0.403, p = 0.000, n = 76), TFe (r = -0.508, p = 0.000, n = 76) and alkalinity (r = -0.233, p = 0.042, n = 76).

Groundwater BCP rates were directly related to GLU activities (E_C) but not to the sum of all EEA values; the regression BCP *vs* E_C was significant (r^2 = 0.616, p = 0.000, F = 45.219, n = 76, slope = 0.414), whereas the regression BCP *vs* total EEA was not significant (r^2 = 0.255, p = 0.107, F = 2.263, n = 88, slope = 0.383). At the same time, BCP rates were directly related to the term $E_T/(1+E_N/E_C+E_P/E_C)$ (r^2 = 0.430, p = 0.001, F = 12.776, n = 88, slope = 0.841), although the regressions BCP *vs* E_C/E_N and BCP *vs* E_C/E_P were not statistically significant (r^2 = 0.140, p = 0.288, F = 1.152, n = 60, slope = 0.157 and r^2 = 0.178, p = 0.515, F = 0.427, n = 71, slope = 0.179, respectively).

DISCUSSION

EEA measured in this study demonstrate that planktonic microorganisms living in the groundwaters of the Doñana aquifer system (SW, Spain) are not dead or compromised cells, as some authors have

suggested (Ghiorse and Wilson, 1988; Pedersen and Ekendahl, 1990; Alfreider *et al.*, 1997). Moreover, our results highlight the important role played by microbial communities in energy fluxes and organic matter cycling in aquifer systems. This study is the first to evaluate EEA in groundwaters in southern Europe, although some previous studies have estimated EEA in groundwaters or sediments of other aquifer systems (Miettinen *et al.*, 1996; Hendel and Marxsen, 1997; Hendel *et al.*, 2001; Lehman and O'Connell, 2002; Cooney and Simon, 2009; Kolehmainen *et al.*, 2009). Mean GLU, LAP and APE activities measured in the groundwaters of Doñana are within the same range of magnitude as those reported for other groundwaters (Miettinen *et al.*, 1996; Hendel and Marxsen, 1997; Hendel *et al.*, 2001; Lehman and O'Connell, 2002), lotic ecosystems (Sinsabaugh *et al.*, 1997; Sinsabaugh and Foreman, 2001) and lentic aquatic systems (Münster *et al.*, 1992; Vaitomaa *et al.*, 2002; Sala and Güde, 2006). By contrast, GLU, LAP and APE activities measured in shallow river or lake sediments are normally several orders of magnitude higher than those found in Doñana (Marxsen and Schmidt, 1993; Sinsabaugh and Findlay, 1995; Álvarez, 2002; Fischer *et al.*, 2005; Wilczek *et al.*, 2005; Sala and Güde, 2006; Zhou *et al.*, 2007), probably because higher organic matter concentrations in sediments compared to groundwaters result in higher EEA (Wilczek *et al.*, 2005). However, it is difficult to compare PHO activity levels found in this aquifer system to those reported in other ecosystems because, to the best of our knowledge, this is the first study to measure PHO activity in groundwaters. In fact, Sinsabaugh (2010) estimated that less than 150 published papers include quantitative measurements of PHO activities from environmental samples. Any case, PHO activity values estimated in sediments of the Hudson river estuary (Sinsabaugh and Findlay, 1995) and in surface waters of a boreal river (Sinsabaugh and Linkins, 1990) were higher than those observed in the groundwaters of Doñana.

It has generally been assumed that most extracellular enzymes are of bacterial origin (Hoppe *et al.*, 1988; Chróst, 1989; Chróst, 1992). In fact, LAP activity is exclusively associated with heterotrophic bacteria (Chróst, 1992). However, some studies have reported ambiguous relationships between EEA and microbiological variables such as bacterial abundance and biomass, concluding that some enzymes (principally phosphatases) may have other origins (Hoppe, 2003; Vrba *et al.*, 2004). In the Doñana aquifer system, only PHO activity values were positively correlated with bacterial abundance, cell biomass and bacterial biomass; moreover, microscope counts showed no eukaryotic organisms (Velasco Ayuso *et al.*, 2009a; Velasco Ayuso *et al.*, 2009b). Therefore, we assume that the extracellular enzyme values estimated were mostly of prokaryotic origin. It is reasonable to find no significant relationships between EEA and microbiological variables such as bacterial abundance or cell biomass because EEA data reflect the active resident microbial community (Coolen and Overmann, 2000; Sinsabaugh and Foreman, 2001), whereas some microbiological variables such as bacterial abundance and cell biomass reflect the entire microbial community (including dead and non-active bacteria, which neither produce nor release extracellular enzymes). Not all planktonic bacteria in shallow sediments of aquifer systems are active (Alfreider *et al.*, 1997; Kieft *et al.*, 1998; Haglund *et al.*, 2002; Lehman and O'Connell, 2002; Goldscheider *et al.*, 2006; Velasco Ayuso *et al.*, 2010a). As a consequence, significant correlations between enzymatic activities and variables reflecting microbial activity levels (*e.g.*,

AMB, BCP or BGR) might be more plausible. Like those of Miettinen *et al.* (1996), our results corroborate these hypotheses.

Three of the four extracellular enzymes assayed in this work (GLU, LAP and APE) are among the most commonly studied in aquatic systems and are generally the most active in space and time (Münster *et al.*, 1992). It is usually recognized that the synthesis of extracellular enzymes is expensive for microorganisms, especially in terms of N. As a consequence, the presence of directly utilizable substrates in the environment usually inhibits the production of hydrolases at the cellular level to save nutrients and energy (Chróst, 1992; Sinsabaugh *et al.*, 1997). GLU is primarily controlled by induction-repression mechanisms regulated by the concentrations of polymeric saccharides and easily available carbon substrates (Miettinen *et al.*, 1996). This enzyme can be rapidly induced and applied to qualitatively poor but time-persistent glycoside-rich substrates (Chróst, 1992; Misic *et al.*, 2002). The piezometer psalsol, which is located on the southeastern shore of Santa Olalla shallow lake (Figure 6.1) and considered to be outflowing from a hydrological point of view (Sacks *et al.*, 1992; Coleto, 2003) (Table 6.1), showed significantly higher GLU activities than most wells during winter 2004, spring 2004 and winter 2005 (HSD tests, $p \leq 0.023$). Moreover, this piezometer showed significantly lower GLU values during autumn 2004 than during winter 2004 and spring 2004 (HSD tests, $p \leq 0.006$). An important phytoplankton bloom usually takes place in Santa Olalla shallow lake during late summer and early autumn (López-Archilla *et al.*, 2004). The decrease in GLU activity in psalsol during autumn 2004 compared to winter 2004 and spring 2004 suggested that the GLU might be repressed by the presencee of easily assimilabe organic carbon, which might be associated with the beginning of algal bloom. Seasonal measures of GLU activities in this area of the Doñana aquifer system are similar to those found by Miettinen *et al.* (1996) in groundwater samples during bank filtration of lake water in Finland. The piezometer po1d, which is located on the southwestern shore of Dulce shallow lake (Figure 6.1), has been described as an inflowing well (Coleto, 2003); this borehole showed a temporal pattern of GLU activities similar to that found in psalsol, with statistically lower GLU activity during summer 2004 than during winter 2004 or spring 2004 (HSD tests, $p \leq 0.000$). As a consequence, the GLU data seem to indicate that the influence of some ecological processes carried out the surface waters of Dulce shallow lake on groundwaters may be more important than previously thought in this area of the aquifer system. The influence of such ecological processes on the groundwaters in po1d strongly depends on the hydrological cycle, being more important in a wet hydrological cycle (such as 2003/2004) than in a dry one (Coleto, 2003). In fact, a very similar temporal pattern was observed in wells po1d and psalsol when all EEA values were taken into account (Figure 6.6), probably reflecting some similarities in the organic matter sources that fuel the metabolism of the microbial communities inhabiting both piezometers. Some deep boreholes, such as SO2a, SO2b and S56, showed very low GLU activities, probably due to low concentrations of easily degradable polymeric compounds, as it has been observed previously by Hendel and Marxsen (1997) in deep groundwaters in Germany. On the other hand, high GLU activities found in wells 39S2 and 39S1 (mainly in summer 2004 and autumn 2004) may be explained by the presence of polymeric saccharides rich in β-bonds. The deep, ascending groundwater flows that supply these boreholes

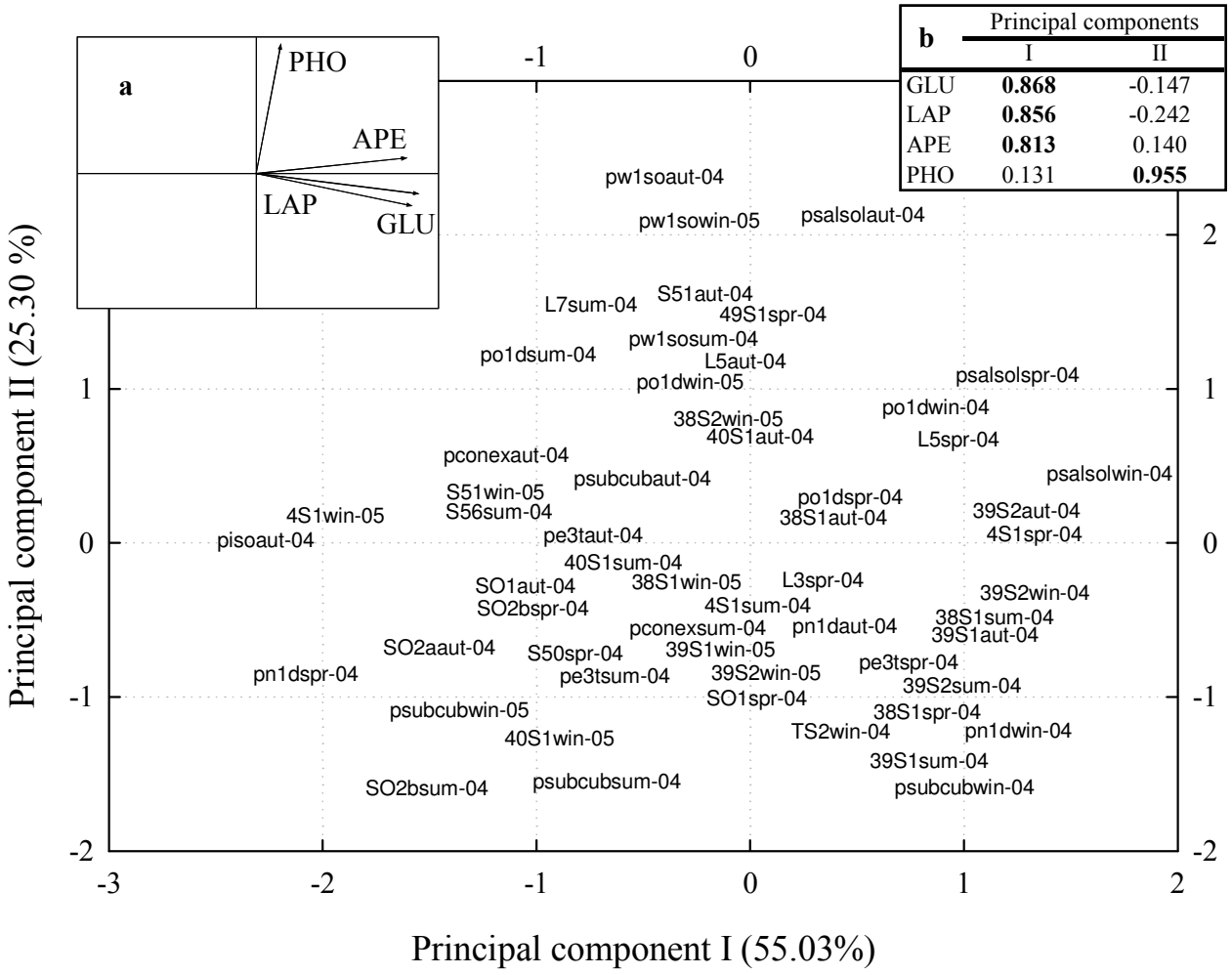

Figure 6.6 Positions of the 55 piezometers in which the activities of four extracellular enzymes were measured in the reduced space of the first two principal components as determined by principal component analysis (PCA). The figure shows the variables projected in the plane determined by the first two principal axes (a) and the factor loadings of these descriptors on both axes (b) (boldface type indicates major factor loadings in each axis) (winter 2004, win-04; spring 2004, spr-04; summer 2004, sum-04; autumn 2004, aut-04; winter 2005, win-05) (GLU, ß-D-glucosidase; LAP, leucine aminopeptidase; APE, alkaline phosphatase; PHO, phenol oxidase).

with water wash and erode deep edaphic formations rich in carbon compounds (Coleto, 2003). Figure 6.6 shows that the positions of piezometers such as 39S2 or 39S1 along principal component I are closer to the position of psalsol than to those of SO2a, SO2b and S56, probably reflecting differences in GLU activity, among other measured EEA.

LAP is considered to be an N-acquiring enzyme (Sinsabaugh *et al.*, 1997) involved in the final step of protein degradation (Wilczek *et al.*, 2005). There are other aminopeptidases, but assays of environmental samples generally show the greatest activities towards leucine- and alanine-linked substrates. Therefore, LAP is widely used as an indicator of peptidase potential (Sinsabaugh *et al.*, 2008). Although some studies have found that LAP activity is induced by low nitrogen conditions and inhibited by inorganic nitrogen (Chróst, 1992), most studies have shown no clear relationships between LAP and inorganic nitrogen compounds (Sinsabaugh *et al.*, 1997; Foreman *et al.*, 1998; Coolen and Overmann, 2000; Findlay *et al.*, 2001; Vaitomaa *et al.*, 2002; Findlay and Sinsabaugh, 2003). The interpretation of LAP activity is complex because this enzyme has a dual role, being

important in both carbon degradation and nitrogen acquisition (Sinsabaugh *et al.*, 1997; Foreman *et al.*, 1998; Findlay *et al.*, 2001; Findlay and Sinsabaugh, 2003). If LAP is acting primarily to provide amino acids to fuel cellular metabolism, then increasing availability of inorganic N should have little effect on its activity. In contrast, if LAP is acting to generate nitrogen, then the presence of inorganic nitrogen should depress its activity (Findlay and Sinsabaugh, 2003). We found no significant relationships between LAP and inorganic forms of nitrogen (ammonium or nitrate). Consequently, we propose that LAP is more active in carbon processing than in nitrogen acquisition in the groundwaters of Doñana. Nevertheless, it is important to note that LAP was the only N-acquiring enzyme whose activity was estimated in this study, while other enzymes, such as β-1,4-N-acetylglucosaminidase, are also important in the acquisition of inorganic nitrogen from dissolved and particulate organic matter (Álvarez, 2002). The piezometer psalsol showed statistically higher LAP activity values than most of the other piezometers during winter 2004 (HSD tests, $p \leq 0.005$), and the temporal pattern of its LAP activity was similar to that observed for GLU, displaying lower activity in autumn 2004 than in winter 2004 and spring 2004 (HSD tests, $p \leq 0.004$). These results suggest that the temporal pattern of LAP activities in well psalsol was controlled, among other factors, by organic compounds of algal origin that were generated in Santa Olalla shallow lake and subsequently transported to the aquifer system by hydrological flows. However, GLU activities were always significantly higher than LAP activities in psalsol (HSD tests, $p \leq 0.024$), probably due to an important presence of carbon compounds with a large number of β-bonds broken by GLU. Moreover, it is generally accepted that nitrogen compounds of algal origin are easily assimilated by bacteria without using extracellular enzymes (Álvarez, 2002). The GLU/LAP ratio changed throughout the year in psalsol, being higher during winter 2004 and spring 2004 than during autumn 2004. This ratio has observed to be higher during production processes and lower during degradation/consumption events (Misic *et al.*, 2002; Alonso-Sáez *et al.*, 2008). Productivity rates are high in the shallow lakes of the Doñana aquifer system during late summer and early autumn (López-Archilla *et al*., 2004); thus, the microbial communities inhabiting outflowing groundwater around Santa Olalla shallow lake displayed a similar pattern of GLU/LAP ratios to that reported for marine waters by Misic *et al.* (2002) and Alonso-Saéz *et al.* (2008). However, it is important to emphasize that the consequences of the accumulation of organic compounds after the algal bloom in Santa Olalla shallow lake appeared in the groundwater with a certain time lag. In general, GLU activities were significantly higher than LAP activities in most wells and seasons, as it has been described in other aquifer systems (Lehman and O'Connell, 2002). This result suggests that N is probably not a limiting factor determining BCP rates in the microbial communities of these groundwaters and reinforces our view of the ecological role played by LAP as a carbon-processing enzyme rather than as a nitrogen-acquiring enzyme. Groundwater samples from some deep wells, such as 39S2, 38S1 and 39S1, were rich in inorganic nitrogen compounds (Coleto, 2003; Velasco Ayuso *et al*., 2009a; Velasco Ayuso *et al*., 2009b) but showed significantly higher LAP activities than most other piezometers in summer 2004 and autumn 2004 (HSD tests, $p \leq 0.034$), demonstrating again that LAP activity was probably related more to carbon processing than to nitrogen acquisition. LAP activity levels in groundwater samples from some other deep wells, such

as SO2b and S56, reinforce this conclusion because these groundwaters were usually rich in inorganic nitrogen compounds and poor in carbon compounds (Coleto, 2003; Velasco Ayuso *et al*., 2009a; Velasco Ayuso *et al*., 2009b). Considering the great heterogeneity of aquifer systems, it is likely that several factors acted simultaneously to induce or repress LAP activity in the groundwaters of Doñana.

APE is a member of a whole *bunch of enzymes*, characterized by different half saturation constants, temperatures and pH (Hoppe, 2003). Several studies have shown that the regulation of APE is primarily mediated by the external concentration of inorganic phosphorus (Hoppe, 2003). We found a significant, positive relationship between TP and APE activities, demonstrating that the presence of polymeric compounds rich in phosphate can enhance the activity of this enzyme. A similar relationship between APE and TP has been described in marine waters (Williams and Jochem, 2006) and river sediments (Wilczek *et al.*, 2005). Thus, microbial communities in the Doñana aquifer system probably used APE as a phosphorus-acquiring enzyme. However, we found no significant relationships between SRP and APE. This result was unexpected because high inorganic phosphorus availability usually represses the activity of APE (Findlay and Sinsabaugh, 2003), and some wells usually showed high SRP concentrations (Velasco Ayuso *et al*., 2009a; Velasco Ayuso *et al*., 2009b). Two explanations have been proposed. First, SRP can enhance the activity of APE when the enzyme is used to access organic phosphorus compounds to meet carbon demands (Hoppe, 2003; Williams and Jochem, 2006). Second, most of the SRP is not biologically available due to the formation of complexes with iron compounds and other chemical processes (Marxsen and Schmidt, 1993; Hoppe, 2003). High concentrations of iron in the groundwaters of Doñana may result in the adsorption of SRP and other inorganic phosphorus compounds, reducing the availability of phosphorus to microbial communities, as it has been proposed by Coleto (2003). Boreholes 39S2, 38S1 and 39S1 showed significantly higher APE activities than most other wells during most seasons (HSD tests, $p \leq 0.037$) and also showed some of the highest BCP rates measured (Velasco Ayuso *et al.*, 2010). Therefore, APE activities were usually highest in wells that also showed high BCP rates and phosphorus demands, as it has been observed in other aquifer systems (Miettinen *et al.*, 1996) and rivers (Wilczek *et al.*, 2005). In fact, we found a significant, positive relationship between APE activities and BCP rates. However, the piezometer psalsol showed statistically lower APE rates during autumn 2004 than during winter 2004 and spring 2004 (HSD tests, $p \leq 0.006$), although its highest BCP rates were measured in autumn 2004 (Velasco Ayuso *et al.*, 2010). At the same time, this well also showed lower APE values than piezometers 39S2, 38S1 and 39S1 during summer 2004 and autumn 2004, probably due to the adsorption of inorganic phosphorus compounds to iron, which displayed high concentrations in this well (Velasco Ayuso *et al.*, 2009a). The presence in the groundwaters of psalsol of easily assimilable inorganic phosphorus compounds that originated in the surface waters of Santa Olalla shallow lake after algal blooms may explain the temporal pattern of APE, as described above for GLU and LAP. Moreover, polyphenols, which were abundant in the groundwaters of piezometer psalsol (Coleto, 2003), can also affect APE activities by complexing metals required by the enzyme (Boavida and Wetzel, 1998). The different positions of psalsol, 39S2, 38S1 and 39S1 along principal component I in the

PCA (Figure 6.6) can probably be explained by the different APE activities found in these four wells, among other reasons.

PHO activity is related to the degradation of polyphenols (Sinsabaugh *et al.*, 2008). Although estimated in only a few studies, PHO activity is ecologically important because it mediates key ecosystem functions, such as degradation, humification, carbon mineralization and dissolved organic carbon export (Sinsabaugh, 2010). The primary role of PHO is to depolymerize recalcitrant phenolic compounds to facilitate the action of hydrolases, such as GLU, LAP and APE, because these phenolic compounds are inherently toxic (Sinsabaugh, 2010) and can inhibit the action of such hydrolases (Pind *et al.*, 1994), especially phosphatases (Boavida and Wetzel, 1998). Alternatively, microbial communities can use PHO to obtain carbon and nutrients during the processing of recalcitrant organic matter (Sinsabaugh, 2010). In this sense, we found a significant, positive correlation between TP and PHO. PHO activities generally show greater spatiotemporal variability than other hydrolases, which may explain the weak correlations found between them (Sinsabaugh and Follstad Shah, 2010b). In fact, in the groundwaters of Doñana, PHO was the only enzyme that showed no significant correlation with any of the other three. The apparent independence of PHO and hydrolase activities suggests that the controls on their expression, activity and turnover differ between them at the community and ecosystem scale (Sinsabaugh and Follstad Shah, 2010b). Boreholes po1d and psalsol showed the highest PHO values during most seasons. The piezometer psalsol, which is clearly influenced by the surface waters of Santa Olalla shallow lake, is an outflowing borehole that always shows high concentrations of polyphenolic compounds. Therefore, it is not surprising to find high PHO activities in this piezometer. Measured PHO activities in the piezometer po1d demonstrate again that this borehole was probably influenced by the surface water of Dulce shallow lake. Other piezometers, such as pw1so, L5, L7, 38S2 and 49S1, showed high PHO activity values in most seasons, with no significant differences among them (HSD tests, $p \geq 0.378$). However, PHO activities in these piezometers differed significantly from those of most other piezometers (HSD tests, $p \leq 0.043$). Moreover, all of these piezometers occupy similar positions along principal component II (Figure 6.6). Boreholes pw1so and 38S2 may be influenced by surface waters from Santa Olalla shallow lake, especially during wet hydrological cycles, as proposed by Coleto (2003). Piezometers L5, L7 and 49S1 are located in parts of the aquifer system where the influence of surface waters rich in organic matter from terrestrial sources is important. Low PHO activities found in deep wells, such as 39S1, SO2a, SO2b, S56 and SO1, suggest a low concentration of recalcitrant carbon compounds in deep groundwaters. In fact, the ratio GLU/PHO, considered to be inversely related to the relative abundance of recalcitrant carbon, was < 1 during all seasons in most piezometers, except in some deep wells (such as 39S1 and SO1) and in boreholes fed by deep, ascending groundwater flows (such as piso). These results suggest that polyphenolic compounds mostly originate and are processed in the first ten to fifteen meters of the Doñana aquifer system.

Once extracellular enzymes are released, their activities are usually controlled by environmental variables. One mode of control is exerted by physical and chemical variables, such as temperature and pH (Sinsabaugh *et al.*, 1997). In our study, taking into account that only PHO

activities showed significant, positive relationships with T, it appears that T was not a key factor controlling EEA the groundwaters of Doñana. Álvarez and Guerrero (2000) have reported that T was not the primary factor controlling the decomposition rates of particulate organic matter mediated by extracellular enzymes in the sediments of two shallow ponds located in Doñana, probably because the physiological optimum temperatures for EEA are usually far from the *in situ* conditions of natural systems (Münster *et al*., 1992). Sinsabaugh and Follstad Shah (2010a) have found that GLU activities, although temporally dynamic, are not directly related to seasonal temperature changes. Moreover, EEA values were assayed here at a standard temperature, which facilitates comparisons but obscures the effects of ambient temperature on relationships (Sinsabaugh and Follstad Shah, 2010a). In most wells, pH was more or less constant and close to neutral throughout the two years of our study (Velasco Ayuso *et al*., 2009a; Velasco Ayuso *et al*., 2009b). Like T, pH was not significantly correlated with EEA values in Doñana. Thus, pH must not be a key factor controlling EEA. The direction of the dependence of EEA on pH is not well established, and both positive (Wilczek *et al*., 2005) and negative correlations (Zhou *et al*., 2007) between EEA and pH have been described in microbial communities of different ecosystems.

GLU/LAP and GLU/APE relationships can respectively indicate N and P availability for microbial communities, reflecting the *microbial perception* of both nutrients (Álvarez, 2002). These ratios depend not only on the presence of organic or inorganic forms of N and P but they are linked to both microbial metabolism and environmental resource availability through the relationships defined in equations [1] and [2] (see materials and methods). Sinsabaugh *et al.* (2009) consider EEA ratios to represent an intersection of stoichiometric and metabolic theories, recently applied to ecological studies, because enzyme expression is a product of cellular metabolism that is specifically regulated by environmental nutrient availability. These relationships can vary among seasons due to seasonal changes in organic matter and nutrient sources, as proposed by Sinsabaugh and Follstad Shah (2010a) and observed by Wilczek *et al*. (2005) in river sediments. Evidences for these relationships are largely circumstantial because although EEA and elemental ratios of organic and inorganic nutrients are easily and frequently measured, coincident measures of TER, B, A and GE are not common (Sinsabaugh *et al.*, 2010). In this study, when the data from all piezometers were analyzed together, regressions of normalized to BCP lnGLU *vs* lnLAP and lnGLU *vs* ln APE showed significantly higher slopes during summer 2004 than during the other seasons. However, we found no significant differences between regressions of normalized to BCP lnGLU *vs* lnLAP and lnGLU *vs* ln APE during any season (Table 6.2). These differences might indicate variations in the stoichiometry of N and P acquisition relative to C. Bearing in mind the slopes of normalized to BCP lnGLU *vs* lnLAP and lnGLU *vs* lnAPE regressions, and the low values of LAP and APE activities in most wells, it seems that the microbial communities had easier access to labile N and P compounds during summer 2004. If the resource supply in the Doñana aquifer system was stable, its microbial community would reach a state in which C, N and P would be co-limiting (Sinsabaugh and Follstad Shah, 2010b), and the slopes would be similar among seasons. This was not the case in the groundwaters of Doñana, a very dynamic habitat. These results should be however interpreted with caution because not all wells were sampled during all seasons. Although aquifer systems have

usually been viewed as oligotrophic environments (Ghiorse and Wilson, 1988; Gounot, 1996), the groundwaters of Doñana show important concentrations of both carbon and nutrients, as it has been reported by Coleto (2003) and Velasco Ayuso *et al*. (2009a; 2009b). Moreover, regressions of normalized to BCP lnGLU *vs* lnLAP and lnGLU *vs* ln APE also showed slopes < 1, suggesting that carbon was not a limiting factor during any season (Table 6.2). Considering that C is not a limiting factor in the Doñana aquifer system, we infer that extracellular enzyme ratios varied according to differences in N and P availabilities, but always to maximize bacterial carbon production rates. Although we found no significant differences, P was apparently more limiting than N on BCP rates in Doñana groundwater throughout the year; the regressions of normalized to BCP lnGLU *vs* lnAPE were always lower than the regressions of normalized to lnGLU *vs* lnLAP (Table 6.2). Moreover, normalized to BCP lnGLU *vs* lnAPE regression negatively correlated with TP, whereas normalized to BCP lnGLU vs lnLAP regression showed no significant relationships with inorganic nitrogen forms (reinforcing our view of LAP as a C-acquisition enzyme). Most wells also showed GLU/APE ratios < 1 during most seasons. However, we note again that LAP was the only N-acquiring enzyme whose activity was estimated in this study, and some other enzymes, such as β-1,4-N-acetylglucosaminidase, are also important for the acquisition of nitrogen compounds and were not measured. The enzyme stoichiometry can be thought as a trade-off among growth efficiency, N- and P-assimilation efficiency and relationships among nutrients in microbial biomass to maintain and maximize BCP rates in microbial communities. The spatiotemporal variability in enzyme ratios found in this study is consistent with several conceptual models for the functional organization of microbial communities, including optimal resource allocation and nutrient co-limitation (Sinsabaugh *et al.*, 2010). The MARCIE model (partially supported by the results of this study) shows that BCP rates are directly related to GLU activities in Doñana groundwaters and to the right-hand term of equation 3 (see materials and methods section), reflecting an allocation strategy among C, N and P enzymes in response to changes in organic matter sources. This allocation strategy is controlled by the metabolic and stoichiometric ratios proposed by Sinsabaugh *et al*. (2009).

Table 6.2 Seasonal and total regression analyses for normalized to BCP extracellular enzyme activities (winter 2004, win-04; spring 2004, spr-04; summer 2004, sum-04; autumn 2004, aut-04; winter 2005, win-05)

	Enzymes	n	r^2	slope	95% CI	intercept	p
win-04	GLU *vs* LAP	9	0.737	0.718	0.329—1.107	3.421	0.024
	GLU *vs* APE	9	0.762	0.579	0.377—0.781	4.223	0.010
	GLU *vs* PHO	13	0.535	0.407	-0.020—0.835	5.720	0.060
spr-04	GLU *vs* LAP	12	0.701	0.746	0.411—1.081	2.368	0.011
	GLU *vs* APE	13	0.491	0.514	0.108—0.921	3.549	0.049
	GLU *vs* PHO	13	0.262	0.218	-0.315—0.752	6.074	0.387
sum-04	GLU *vs* LAP	14	0.806	0.979	0.727—1.231	1.076	0.001
	GLU *vs* APE	17	0.700	0.818	0.559—1.078	0.557	0.002
	GLU *vs* PHO	18	0.359	0.277	-0.105—0.658	2.579	0.143
aut-04	GLU *vs* LAP	14	0.332	0.329	-0.259—0.917	3.559	0.247
	GLU *vs* APE	19	0.432	0.439	-0.030—0.909	1.823	0.065
	GLU *vs* PHO	19	0.316	0.269	-0.144—0.683	2.115	0.187

Table 6.2 Continued

	Enzymes	n	r^2	slope	95% CI	intercept	p
win-05	GLU *vs* LAP	11	0.620	0.733	0.233—1.233	0.674	0.042
	GLU *vs* APE	12	0.698	0.677	0.387—0.967	0.674	0.012
	GLU *vs* PHO	13	0.690	0.535	0.363—0.707	0.717	0.009
total	GLU *vs* LAP	60	0.816	0.972	0.701—1.021	1.395	0.000
	GLU *vs* APE	70	0.747	0.861	0.764—1.080	1.140	0.000
	GLU *vs* PHO	76	0.451	0.529	0.387—0.671	1.206	0.000

CONCLUSIONS

Local and regional hydrogeological flows, which transport organic matter and nutrients of different origins, support the activity of microbial communities in the Doñana aquifer system, maintaining microbial BCP rates. BCP rates are directly related to carbon obtained by means of GLU. However, microbial communities must also obtain N and P. GLU/LAP and GLU/APE ratios varied among seasons, suggesting that microbial communities may vary their response more to differences in organic matter quality than to differences in organic matter quantity, as it was proposed by Wehr *et al.* (1999). The allocation strategy among C, N and P enzymes shown by microbial communities inhabiting the groundwaters of Doñana in response to changes in organic matter sources is related to both metabolic and stoichiometric relationships. It appears that P is more limiting than N in the Doñana aquifer system, probably because the adsorption of inorganic phosphorus compounds to iron compounds, which are abundant in Doñana, makes the direct acquisition of P difficult. Moreover, PHO activity levels demonstrate the major role of the phenol oxidase enzyme in the degradation of polyphenolic compounds to facilitate the action of hydrolases to obtain of C, N and P, except in some deep areas of the aquifer system. We therefore propose that aquifer systems, at least their shallower areas, have a similar functional role with respect to their associated aquatic and terrestrial systems to that played by hyporheic systems and their associated rivers. Our results prove that the degradation process of organic matter started in the sediments of the shallow lakes located in Doñana (Álvarez, 2002) continues in the groundwaters of the aquifer system, with the objective of recycling organic carbon and nutrients, channelled through microorganisms, for transporting them later to higher trophic levels by means of hydrological flows (Coleto, 2003).

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Capítulo 7. La diversidad de las comunidades microbianas del acuífero de Doñana

7. ARCHAEAL AND BACTERIAL COMMUNITY COMPOSITION OF A PRISTINE COASTAL AQUIFER IN DOÑANA NATIONAL PARK, SPAIN

Aquatic Microbial Ecology (2007), 47, 123-139

ABSTRACT

We have studied the biological activity and the prokaryotic diversity associated with two samples of different physicochemical characteristics in a coastal pristine aquifer at the Doñana National Park (SW, Spain). Sulphate reduction, denitrification and iron-metabolising activities were detected in the aquifer, as well as different enzymatic activities related with the degradation of labile and complex pools of organic matter. Prokaryotic diversity was assessed by environmental 16S rRNA gene amplification, cloning and sequencing. Bacterial diversity was greater in the shallower aquifer sample (49S1, 14 m depth) including members of the *Proteobacteria, Acidobacteria, Planctomycetes, Nitrospirae*, some divergent lineages of the candidate divisions SPAM, OP3, OP11, 'Endomicrobia' or Termite Group 1 and a novel division-level group including denitrifying bacteria associated to anaerobic methane oxidising archaea, whereas only *Proteobacteria* and *Firmicutes* were detected in the deeper sample (S56, 80 m depth). By contrast, archaea seemed much more diverse in the deeper aquifer sample, with members of the *Methanomicrobiales*, ANME2-related *Methanosarcinales* and other divergent lineages, whereas only Group I *Crenarchaeota* were detected in the shallower sample. *Betaproteobacteria* were the most abundant and diverse group in both sample libraries, together with the *Gammaproteobacteria* in the deeper and more saline S56 sample. We detected microorganisms potentially involved in carbon, sulphur and nitrogen cycling. Interestingly, members of both aerobic (*Alpha*- and *Gammaproteobacteria*) and anaerobic methane oxidisers (ANME2 archaea) were found in the same aquifer sample.

7. COMPOSICIÓN DE LAS COMUNIDADES DE BACTERIAS Y ARQUEAS DE UN SISTEMA ACUÍFERO COSTERO Y PRÍSTINO EN EL PARQUE NACIONAL DE DOÑANA, ESPAÑA

Aquatic Microbial Ecology (2007), 47, 123-139

RESUMEN

La diversidad de procariotas y la actividad biológica han sido estudiadas en dos muestras de diferentes características fisicoquímicas en el sistema acuífero prístino del Parque Nacional de Doñana (SW, España). Actividades microbianas desnitrificantes, sulfatorreductoras y relacionadas con el hierro fueron detectadas en el acuífero, así como diferentes actividades enzimáticas relacionadas con la degradación de la materia orgánica tanto lábil como recalcitrante. La diversidad de procariotas fue estimada a través de técnicas de amplificación, clonación y secuenciación de genes de ARNr 16S. La diversidad bacteriana fue mayor en la muestra del piezómetro somero (49S1, 14 m), incluyéndose miembros de *Proteobacteria*, *Acidobacteria*, *Planctomycetes*, *Nitrospirae*, algunas líneas divergentes de las divisiones candidatas SPAM, OP3, OP11, 'Endomicrobiota' o Grupo 1 Termita y un nuevo nivel de división que incluye bacterias desnitrificantes asociadas a arqueas anaeróbicas oxidadoras de metano, mientras que únicamente miembros de *Proteobacteria* y *Firmicutes* fueron detectados en la muestra del piezómetro profundo (S56, 80 m). Por el contrario, las arqueas fueron mucho más diversas en la muestra del piezómetro profundo, con miembros de *Methanomicrobiales*, miembros relacionados con ANME-2 *Methanosarcinales* y algunas otras líneas divergentes, mientras que solamente miembros del Grupo I *Crenarchaeota* fueron detectados en la muestra del piezómetro más somero. *Betaproteobacteria* fue el grupo más abundante en ambas muestras, junto con *Gammaproteobacteria* en la muestra del piezómetro más profundo y salino S56. Se han detectado microorganismos potencialmente relacionados con los ciclos del carbono, azufre y nitrógeno. El hecho de que se hayan encontrado en la misma muestra microorganismos oxidadores de metano tanto aerobios (*Alpha*- y *Gammaproteobacteria*) como anaerobios (arqueas ANME2) es muy interesante.

INTRODUCTION

In recent years, studies on aquifer microbiology received an increasing interest, as there is an important concern about the quality of natural water resources (Leclerc and Moreau, 2002). Underground aquifers serve as important source of potable water. They include shallow, intermediate and deep aquifers, depending on their depth and their active water flow (in the order of meters per day, year and century, respectively). They constitute the natural biotopes of subsurface microbial communities, whose diversity and ecology have also been actively studied in the last years. This is partly due to the development of molecular tools to characterise microbial diversity, particularly 16S rRNA gene-based surveys, as most of these subsurface microorganisms are difficult to retrieve in culture (Ghiorse and Wilson, 1988; Pedersen, 2000; Parkes *et al*., 2005; Schippers *et al*., 2005).

Most of the microbial diversity studies focusing on aquifer communities have been carried out in sites contaminated by different linear and polycyclic hydrocarbons, chlorinated compounds or heavy metals (*e.g.*, Dojka *et al*., 1998; Takai *et al*., 2001; Kleikemper *et al*., 2002). Aquifers contaminated with organic compounds have been investigated to identify indigenous microorganisms involved in natural bioremediation aiming at prospective biotechnological applications. Early molecular diversity studies of hydrocarbon- and chlorinated-solvent contaminated aquifers revealed the presence of microorganisms belonging to known bacterial phyla as well as divergent lineages affiliating to candidate divisions or defining novel candidate-division-level groups (Dojka *et al*., 1998). By contrast to hydrocarbon-polluted aquifers, organic matter is scarce in aquifers associated to deep underground systems, such as deep mines (Pedersen, 2000) or the deep sub-seafloor (Fisk *et al*., 1998; Edwards *et al*., 2005). Microbial life in such systems is thought to be largely based on chemolithoautotrophy and metabolic rates may be extremely low (Parkes *et al*., 2005; Schippers *et al*., 2005).

Compared to contaminated aquifers, pristine sites have been less studied. Nevertheless, reports on the microbial diversity associated with deep aquifers, particularly of sites possessing distinctive physicochemical characteristics such as acidic (Lehman *et al*., 2001), alkaline (Fry *et al*., 1997) or geothermal (Chapelle *et al*., 2002; Kimura *et al*., 2005) subsurface environments exist. Pristine aquifer systems can be very different according to the geological context and among those that have deserved microbial classical and molecular studies, some flow through igneous rocks (granites and basalts) (Pedersen, 2001; Miyoshi *et al*., 2005; Ball and Crawford, 2006), others settle in sedimentary rocks, karstic environments or sandy areas (Hirsch and Rades-Rohkohl, 1990; Shi *et al*., 1999; Detmers *et al*., 2001; Farnleitner *et al*., 2005; Santoro *et al*., 2006).

Also a few comparative studies comparing contaminated and pristine aquifers have been reported (Griebler *et al*., 2002). In a comparative analysis between two different sampling points of the same aquifer, one fuel-contaminated and the other non-polluted, Shi and co-workers (1999) found a higher proportion of *Beta*- and *Gammaproteobacteria* in the contaminated sample. Another comparative study focusing on the long-term effect of benzene on a sandstone aquifer showed a reduced microbial diversity in contaminated compared to control sites. Multivariate statistical

analyses indicated a correlation between the decrease in microbial diversity and anoxia. Rather than the toxic effect of benzene itself, anoxia caused by the microbial degradation of benzene and the decrease of available redox species were the major factors explaining the decline of microbial diversity (Fahy *et al*., 2005).

Although molecular surveys based on 16S rRNA and conserved metabolic genes give an insight on the phylogeny and potential activity of microorganisms that are present in the subsurface, there is little information about their actual metabolic status, as an unknown proportion of the detected phylotypes may be inactive. In this work, we have studied the microbial diversity and activities associated to a pristine, coastal aquifer located in the Doñana National Park (SW, Spain). Doñana is one of Europe's most important wetland reserves and a major site for migrating birds. This area has a typical mediterranean climate (dry and hot summers and little rainfall winters) with some oceanic influences, broad seasonal temperature and precipitation variability, and quite stable annual variability across time. Coastal marshes represent the largest epicontinental aquatic environment in Doñana, together with several shallow lakes (López-Archilla *et al*., 2004), whose integrity and long-term ecological maintenance is assured by groundwater input.

The aquifer of Doñana consists of detritical deposits from the Neogene period, which are covered by quaternary fluvio-marine and aeolian materials. Sands, silty sands and gravels, occasionally inter-layered with fine clay sedimentary materials, are the main components of the system (Trick and Custodio, 2004). Doñana's aquifer is eminently freshwater, although it may be locally affected by seawater intrusion, and from a hydrodynamic point of view, it is confined below the clay material, being phreatic in the sand layers (Custodio *et al*., 1995; Lozano and Palancar, 1995). In general, the phreatic level is adjusted to the topography, with an average of six meters for the water table depth in many different points (Custodio *et al*., 1995; Lozano, 2004). Here we report the study of two samples representing two distinct sub-systems of Doñana's aquifer (49S1 and S56 at 14 and 80 m depth, respectively). Microbial diversity was greater in the shallower site, which had lower mineral content, higher oxygen concentration and showed higher potential hydrolytic activities. However, the deeper site exhibited a relatively wide diversity of *Euryarchaeota* with lineages clearly belonging to the anaerobic methane oxidising archaeal group ANME2. These were identified together with phylotypes of classical aerobic methane oxidising bacteria, suggesting that both, aerobic and anaerobic methane oxidation coexist in the same aquifer samples.

MATERIALS AND METHODS

Sampling and physicochemical measurements

Groundwater samples were collected in July 2004 from the aquifer that feeds Doñana National Park (SW, Spain) using two different piezometers designed as 49S1 and S56. The wells were drilled by the Guadalquivir river Water Authority in the aeolian littoral mantles, a geomorphological formation made up of sand deposition in the form of active and stabilised dunes. The co-ordinates for the two piezometers were X = 721413, Y = 4096827, UTM 29 (49S1) and X = 733070,Y = 4087500, UTM 29 (S56). The piezometer screen intervals were situated at a depth of 14 m (49S1)

and between 74 and 80 m (S56). Groundwater samples were taken with the help of a pneumatic pump (Uwitec, Mondsee, Austria) especially designed to pump the aquifer interstitial water, avoiding its mixture with the water accumulated in the piezometer. The porosity of the sand unit sustaining the aquifer corresponds to 23-38% (Iglesias, 1999). Pumped groundwater was collected from each borehole only once measurements of temperature, dissolved oxygen -DO-, pH and electrical conductivity -EC- had stabilised (Haveman and Pedersen, 2002).

Physical parameters (temperature, DO, pH and electrical conductivity) were measured *in situ* with a WTW 340i handheld multi-parameter device (WTW, Weilheim, Germany) and samples for microbiological analysis were taken (see below). The piezometric level was measured in each piezometer with a water level acoustic indicator (Nordmeyer, Peine, Germany). For chemical determinations (alkalinity, ammonium, nitrate, nitrite, soluble reactive phosphorus -SRP- and total phosphorus -TP- concentration), samples were stored in triplicate in polyurethane or glass bottles, previously treated with 5% HCl and washed with distilled water, and then kept frozen or refrigerated until laboratory analyses were done. Chemical analyses were carried out using standard methods (APHA *et al*., 1989). Alkalinity was measured during the same sampling day. Ferrous and ferric ions, as well as the total iron concentration, were determined in the laboratory by the ferrozine colorimetric method (Viollier *et al*., 2000). Various water samples were collected carefully in sterile containers to prevent possible external contamination and were conditioned in the appropriate ways (see below) for subsequent microscopy, enzymatic and molecular biology analyses in the laboratory.

Biological parameters and enzymatic assays

For epifluorescence microscopy observations, groundwater samples from each piezometer were collected and fixed with formaldehyde (4% v/v final concentration) in 100 mL polyurethane bottles (pre-treated with HCl and rinsed with distilled water, as above) in triplicate; these samples were stored in the dark at 4 ºC until further analysis. Water samples were filtered through 0.2 µm Millipore GTBP Membrane filters (Millipore, Billerica, Massachusetts, USA) and cells counted by epifluorescence microscopy observation after staining with DAPI (4',6-diamidino-2-phenylindole-dihydrochloride) (Fry, 1990). Single cell biovolumes were estimated with the aid of an ocular micrometer under an Olympus IX50 inverted microscope to measure the lengths and widths of cells with a magnification of ×1000 in the same filters where their abundance was determined. Cell biovolumes were calculated assuming that cells were spheres or cylinders with hemispheres on both sides. Linear dimensions were converted to volumetric ones using geometric formulas and the single cell biomass calculated from single cell biovolumes using the allometric model conversion factor: $C = 120 \times V^{0.7}$ (Bölter *et al*., 2002).

Water samples to estimate microbial activity, productivity as deduced from the leucine incorporation rate, enzymatic activity, bacterial functional groups and community diversity studies were collected in sterile glass bottles, stored at 4 °C and transported to the laboratory. Microbial activity was estimated using an ATP-based method on triplicate groundwater samples collected in 20 mL bottles. ATP assays were carried out during the same sampling day. ATP was extracted with

a commercial kit (BioThema, Handen, Sweden) and quantified via the luciferin-luciferase enzyme assay using a Berthold Junior LB9509 luminometer (Berthold Detection Systems, Pforzheim, Germany), with a detection limit of approximately 10×10^{-18} mol ATP. For each assay, ATP was extracted from 50 µL groundwater following the manufacturer's instructions. Duplicate autoclaved water samples were used as controls for each assay. The relative light units (RLU) measured with the luminometer were converted into ATP concentration values using a regression equation calculated with an ATP standard provided in the same commercial kit. To estimate the active biomass, we applied the conversion factor: 1 ngATP L^{-1} = 250 ngC L^{-1} (Karl, 1980).

Microbial productivity was estimated by measuring the incorporation of [^{3}H]leucine into cellular proteins (Kirchman *et al*., 1985). Three replicates and two controls were analysed for each piezometer. [^{3}H]leucine (37 MBq mol^{-1}; Amersham Biosciences, Chalfont St. Giles, UK) was added to 10 mL groundwater to a final concentration of 11 nM, and incubated during 1 hour at 20 ºC. Leucine incorporation was stopped with 1.1 mL of formaldehyde, and samples were frozen (-20 ºC) until protein extraction. Insoluble protein material was precipitated with hot trichloroacetic acid and collected on cellulose filters (Kirchman *et al*., 1985). Incorporated radioactivity was measured after addition of scintillation cocktail in a LKB Wallack 1209 Rackbeta Liquid Scintillation Counter (LKB Instruments, Mount Waverley, Victoria, Australia). Microbial productivity was then estimated according to the equation Productivity = LI × 131.2 × 0.073 × (C/protein) × ID, where LI is the leucine incorporation rate (mol L^{-1} h^{-1}), 131.2 is the molecular weight of leucine, 0.073 is the average percentage of leucine in cell proteins, C/protein is the ratio of cellular carbon to protein (0.86) and ID is the isotope dilution (both external and internal), which is assumed not to have taken place in this study (Simon and Azam, 1989).

The enzymatic activities ß-D-glucosidase, leucine aminopeptidase, alkaline phosphatase and phenol oxidase were assayed by incubation with pNP-ß-D-glucopyranoside, leucine p-nitroanilide, pNP-phosphate and L-3,4-dihydroxyphenylalanine (L-DOPA), respectively. All substrates were obtained from Sigma Chemical Company (Saint Louis, Missouri, USA). Assays for ß-D-glucosidase and phenol oxidase were conducted at 20 ºC in 50 mM acetate buffer, pH 5.0, under previously determined substrate saturating conditions. For alkaline phosphatase and leucine aminopeptidase, assays were conducted in 5 mL reaction volumes with 50 mM Tris-HCl, pH 8.5, under previously determined substrate saturating conditions. In all cases three analytical replicates and two controls were carried out. A more detailed description of the procedures used can be found in Sinsabaugh *et al*. (1994).

Different microbial functional activities revealing the presence of nitrifying, sulphate-reducing, denitrifying and iron-metabolising microorganisms were assayed using BART™ tubes (*Biological Activity Reaction Tests*) (Cullimore, 1993). Tests are done at room temperature and consist of the inoculation of a sample in a culture tube with the particular medium assayed that contains a colour indicator shifting upon a redox change. A ball is placed on top of the water column that restricts the amount of oxygen entering it, so that aerobic organisms grow around the ball and anaerobic organisms grow deep down in the water column. If the type of organisms tested

is present in the sample, the indicator will change of colour, indicating that the targeted reaction has taken place. These tests are semi-quantitative, allowing not only the detection of a given activity but also providing an indication of the level of activity. In our case, the presence of the above functional groups was determined in 10 mL groundwater samples from each piezometer.

DNA extraction, PCR amplification, cloning and sequencing

For molecular diversity estimates, 1 L of each groundwater sample was filtered across 0.22 μm-pore diameter Millipore GTTP filters (Millipore, Billerica, Massachusetts, USA), which were stored at –20 °C until nucleic acids were extracted. Filters were washed extensively with phosphate saline buffer (130 mM NaCl, 10 mM phosphate buffer, pH 7.4, PBS) to resuspend the retained biomass. Subsequently, lysis proceeded by the addition of 80 μg mL^{-1} proteinase K, 1% SDS, 1.4 M NaCl, 0.2% β-mercaptoethanol and 2% CTAB (final concentrations) and the incubation of the samples overnight at 55 ºC. DNA from lysates was extracted sequentially with hot phenol (65 °C), phenol-chloroform-isoamyl-alcohol, and chloroform-isoamyl-alcohol. Nucleic acids were concentrated by salt and ethanol precipitation. Nucleic acids were resuspended in 10 mM Tris-HCl, pH 8.0. 16S rRNA genes were subsequently amplified by PCR using different combinations of bacterial (B-), archaeal (A-) and prokaryotic-specific primers: B-27F (AGAGTTTGATCCTGGCTCAG), A-23FLP (GCGGATCCGCGGCCGCTGCAGAYCTGGTYGATYCTGCC), Ar109F (ACSGCTGCTCAGTAACACGT), A-ANMEF (GGCTCAGTAACACGTGGA), and 1492R (GGTTACCTTGTTACGACTT). PCR reactions were performed under the following conditions: 30 cycles (denaturation at 94 ºC for 15 s, annealing at 50 ºC to 55 °C for 30 s, extension at 72 ºC for 2 min) preceded by 2 min denaturation at 94 ºC, and followed by 7 min extension at 72 ºC. Dimethyl sulfoxide was added to a final concentration of 3-5% to the PCR reaction mix. 16S rRNA gene clone libraries were constructed using the Topo TA Cloning system (Invitrogen, San Diego, California, USA) following the instructions provided by the manufacturers. Three 16S rRNA gene libraries were constructed for the archaea (2 for 49S1 and 1 for S56), and another three for bacteria (2 for 49S1 and 1 for S56). After plating, positive transformants were screened by PCR amplification of inserts using M13R and T7 flanking vector primers, and positive clones sequenced by Genome Express (Meylan, France) with the PCR amplification primers used in each case. Partial sequences (600-800 bp) of good quality were retained for subsequent analyses, 71 and 90 bacterial sequences, and 19 and 21 archaeal sequences from 49S1 and S56, respectively, making a total of 201 sequences (Table 7.2). From these, a total of 75 clones representative of the diversity found (representing groups of sequences > 97% identical) were fully sequenced in order to carry out detailed phylogenetic analyses: 44 and 19 bacterial clones, and 4 and 8 archaeal ones, from 49S1 and S56, respectively.

Biodiversity estimates

Rarefaction curves and different biodiversity indices were estimated from our sequence data using the program DOTUR (Schloss and Handelsman, 2005). Prokaryotic high-quality partial sequences were aligned using ClustalX (Thompson et al., 1997) for 49S1 (14 m depth) and S56 (80 m depth) independently, and the respective distance matrices were generated in Phylip format after excluding

gaps. The resulting matrices were used as input for DOTUR in order to generate rarefaction curves at 97% sequence identity level, corresponding roughly to the species level, lineage-through-time plots, and different species richness indicators considering 97% (species) and 95% (genus level) threshold values for the definition of Operational Taxonomic Units (OTUs) (Table 7.2). The homologous and heterologous coverage of the two sets of libraries (for 49S1 and S56 respectively) as a function of phylogenetic distance was compared using the program LIBSHUFF (Singleton *et al.*, 2001).

Phylogenetic analyses

Alignment and preliminary distance phylogenetic analysis of the 205 partial 16S rRNA gene sequences were done using ClustalX. This allowed the identification of identical or nearly identical sequences and the selection of clones for complete sequencing. The 75 representative clones that were completely sequenced, together with their closest homologues in GenBank (http://ncbi.nlm.nih.gov/) as detected by BLAST (Altschul *et al.*, 1997), were aligned automatically with an alignment containing ~16000 16S rRNA gene sequences using the program BABA (Philippe, personal communication). The multiple alignment was then manually edited using the program ED from the MUST package (Philippe, 1993). Neighbour-joining (NJ) trees were constructed for the different prokaryotic taxa in order to choose representative subsets of sequences for further phylogenetic analyses. Gaps and ambiguously aligned positions were excluded from our analyses using strict criteria, which resulted in alignments of variable length (positions). Four different subsets of 16S rRNA gene sequences were selected to have a good taxonomic coverage of different regions in phylogenetic trees. These subsets were: Archaea (49 sequences, 848 positions), overall bacterial phylogeny including candidate divisions (91 sequences, 1092 positions), *Betaproteobacteria* + *Gammaproteobacteria* (94 sequences, 1238 positions), and *Alphaproteobacteria* + *Deltaproteobacteria* (49 sequences, 1187 positions). These datasets were analysed by maximum likelihood (ML) using TREEFINDER (Jobb, 2002) applying a general time reversible model of sequence evolution (GTR), and taking among-site rate variation into account by using a six-category discrete approximation of a Γ distribution (invariable sites are included in one of the categories). ML bootstrap proportions were inferred using 1000 replicates. Phylogenetic trees were viewed using the program TREEVIEW (Page, 1996). The sequences reported in this study were submitted to GenBank with accession numbers DQ837222 to DQ837296 (Figures 7.3, 7.4 and 7.5).

RESULTS AND DISCUSSION

Physicochemical characteristics of sampling sites

A preliminary study of the physicochemical and biological characteristics of various samples collected from a variety of piezometers placed at 30 different sites and depths in Doñana had revealed the presence of two types of samples that shared, respectively, very similar and homogeneous characteristics (Velasco Ayuso and López-Archilla, unpublished data). They corresponded to two distinct sub-systems in the aquifer, one laying below the sand dunes and

characterised by low-mineral waters and the second, richer in minerals, below the salt marshes. We therefore selected one representative sample of each, namely 49S1 and S56, for further analysis.

Table 7.1 Physicochemical and biological variables measured in the 14 m-deep (49S1) and 80 m-deep (S56) samples collected from the coastal aquifer system of Doñana (T, temperature; SRP, soluble reactive phosphorus; TP, total phosphorus; ND, not determined; ++, +++, increasing growth yield; -, no growth)

	49S1	**S56**
Sampling depth (m)	11.4-14.2	74.0-80.0
T (ºC)	19.2 ± 0.1	22.1 ± 0.1
Phreatic level[1] (m)	3.06	1.64
Dissolved oxygen (mg L^{-1})	1.13 ± 0.06	0.86 ± 0.00
pH	5.17 ± 0.14	7.45 ± 0.03
Electric conductivity (mS cm^{-1})	0.15 ± 0.02	21.01 ± 0.15
Total alkalinity (meq L^{-1})	0.523 ± 0.023	6.000 ± 0.000
[N-NH_4^+] (mg L^{-1})	1.22 ± 0.09	7.78 ± 1.39
[N-NO_2^-] (mg L^{-1})	0.004 ± 0.007	0.000 ± 0.000
[N-NO_3^-] (mg L^{-1})	0.339 ± 0.071	0.431 ± 0.034
[Fe^{2+}] (mg L^{-1})	0.051 ± 0.015	0.122 ± 0.083
[Fe^{3+}] (mg L^{-1}))	0.379 ± 0.242	1.317 ± 0.724
Total [Fe] (mg L^{-1})	0.430 ± 0.255	1.439 ± 0.648
[P-SRP] (mg L^{-1})	0.002 ± 0.000	0.423 ± 0.041
[P-TP] (mg L^{-1})	0.013 ± 0.001	0.521 ± 0.041
Active biomass[2] (gC mL^{-1})	ND	$3.8\ 10^{-7} \pm 1.1\ 10^{-7}$
Cell density (10^7 cell mL^{-1})	9.30 ± 8.74	9.34 ± 8.15
Cell volume (μm^3)	0.78 ± 0.89	1.05 ± 1.04
Cell biomass (fgC)	89.97 ± 72.15	114.62 ± 76.00
C incorporation (10^{-8} g C $h^{-1}L^{-1}$)	2.8 ± 2.0	0.3 ± 0.2
Glucosidase activity[3] (nmol $h^{-1}mL^{-1}$)	0.738 ± 0.557	0.000 ± 0.000
Aminopeptidase activity[3] (nmol $h^{-1}mL^{-1}$)	0.004 ± 0.012	0.001 ± 0.002
Alkaline phosphatase activity[3] (nmol $h^{-1}mL^{-1}$)	0.616 ± 0.182	0.003 ± 0.006
Phenol oxidase activity[3] (nmol $h^{-1}mL^{-1}$)	2.869 ± 1.650	5.348 ± 0.661
Sulphate reduction (BART™ tests)	++	+++
Nitrification (BART™ tests)	-	-
Denitrification (BART™ tests)	++	+++
Iron-metabolising (BART™ tests)	++	+++

[1]Groundwater table
[2]Estimated by the ATP method (Materials and Methods)
[3]nmol of transformed substrate h^{-1} mL^{-1} (Sinsabaugh *et al*., 1994)

We measured various physicochemical parameters and collected samples for biological analyses from the two selected piezometers. Piezometer 49S1 was placed at 14 m depth and approximately 2 km from the shoreline, and S56 at approximately 80 m depth and 0.5 km from shore. The physicochemical characteristics of the two aquifer samples were quite different. The shallower site, 49S1, was more acidic (pH 5.17) and with less alkalinity (0.5 meq L^{-1}), and contained lower inorganic nutrient concentrations, including soluble reactive phosphorous (SRP), nitrite, nitrate, ammonium and iron (Table 7.1). By contrast, S56 had neutral pH (7.45) and higher alkalinity (6 meq L^{-1}), as well as higher concentrations of inorganic nutrients (Table 7.1). The presence of relative high levels of Fe^{3+} in the samples is due to the occurrence of a deeper layer of red sands rich in iron oxides (red formation) that is washed by the up-welling water flux (Trick and Custodio, 2004). The two sites were suboxic, although S56 was closer to anoxia than 49S1 (oxygen concentration of 0.86 mg L^{-1} versus 1.13 mg L^{-1}, respectively). Notably, conductivity values were far higher in S56 (21 mS cm^{-1}) than in 49S1 (0.15 mS cm^{-1}), being intermediate between typical

freshwater and seawater levels (41.5 mS cm^{-1}). This reveals the marine influence due to the greater depth of the S56 piezometer and its closest location to the seashore.

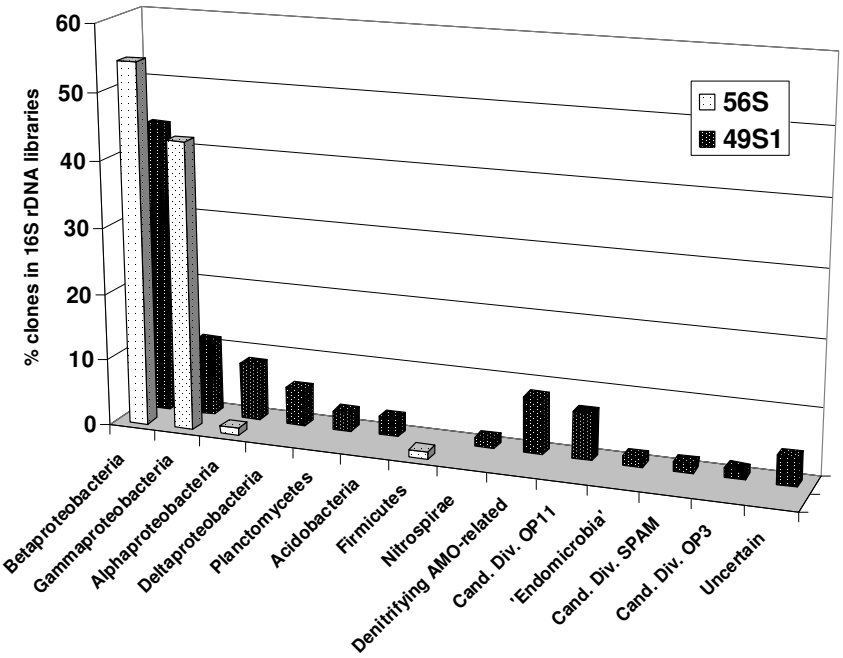

Figure 7.1 Distribution in major phylogenetic groups of 16S rRNA bacterial genes of the 14-deep (49S1) and 80m-deep (S56) samples collected from the coastal aquifer system in Doñana National Park. Denitrifying-AOM-related corresponds to the novel division-level group proposed by Raghoebarsing *et al.* (2006) including the bacterial symbiont of a denitrifying AOM consortium. 'Endomicrobia' is a candidate phylum equivalent to the Candidate Division Termite Group 1 (AOM, anaerobic oxidation of methane; Cand. Div., candidate division).

Biological parameters and enzymatic activities

Cell density was similar in both aquifer samples, although the error bars for these measurements were important due to the small cell size and to the unspecific DAPI staining of some tiny sediment particles suspended in the sample. Despite similar cell densities, both aquifer samples showed different biological activities, as shown by BART^{TM} tests and enzymatic assays. It is important to note that these activities are tested in the laboratory by incubation of aquifer samples and, therefore, they indicate only potential metabolic activities taking place *in situ*. The biological heterotrophic activity in the deepest sample was smaller, as the incorporation rate of [^{3}H]leucine was more important in 49S1 than in S56. By contrast, S56 displayed greater sulphate reduction, denitrification and iron-metabolising activities as revealed by the BART^{TM} tests (see materials and methods, and Table 7.1). The phenoloxidase activity, which is related to the degradation of recalcitrant organic matter fractions (Sanderman and Amundson, 2005), was also higher in S56. On the contrary, 49S1 samples displayed higher phosphatase, aminopeptidase and glucosidase activities (Table A.1), which are involved in the degradation of nucleic acids, polypeptides and linear carbohydrates constituting the easiest accessible fractions of organic matter that are first consumed (labile pool). A higher phenoloxidase activity in deeper aquifer layers would be in agreement with the consumption of simpler organic matter in upper layers, while only the more complex, aromatic-rich, organic compounds percolate to greater depths. Although cell density was similar in both aquifer samples,

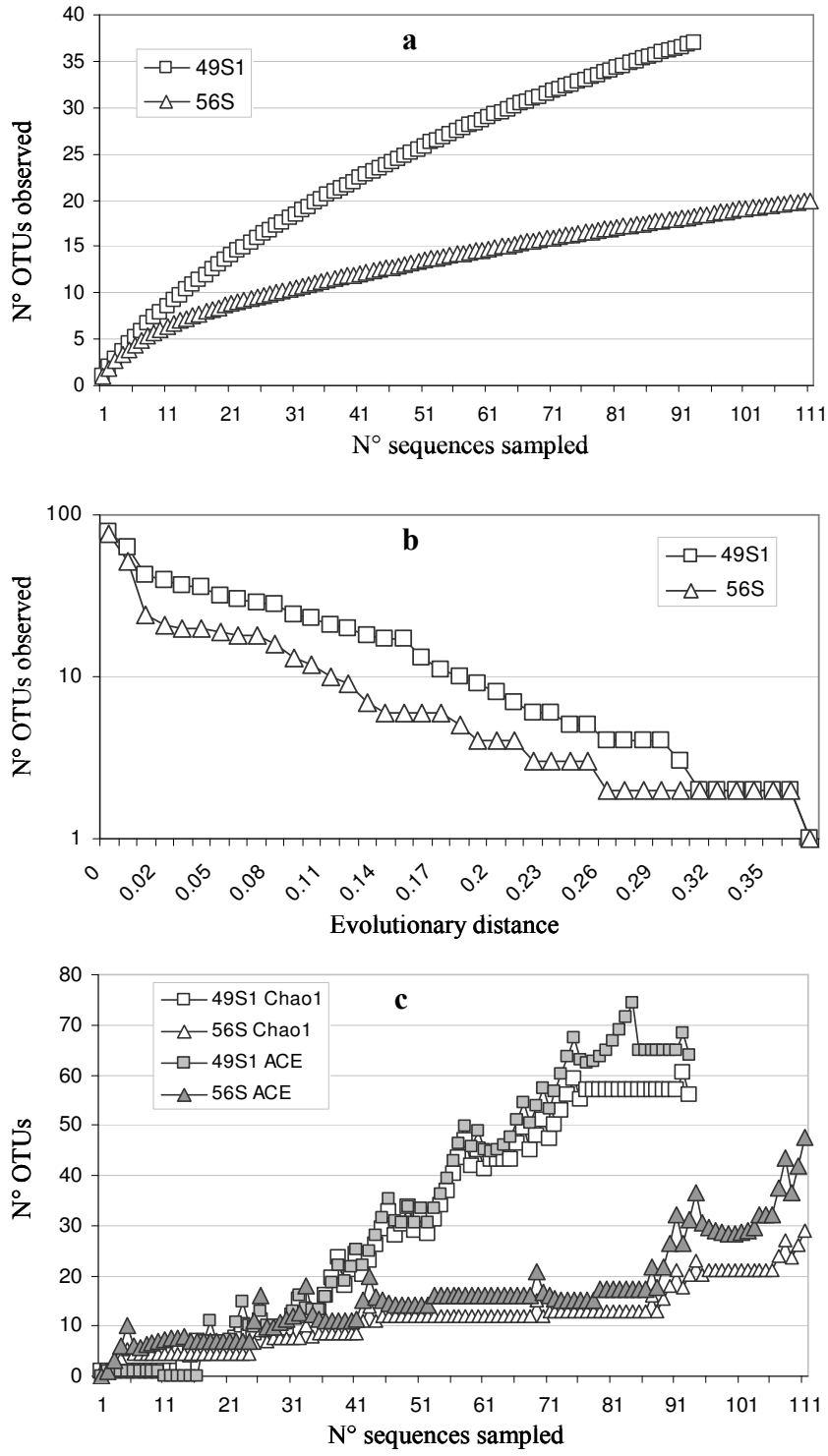

Figure 7.2 Rarefaction curves (a), lineage-trough-time plots (b) and Chao1 and ACE richness estimate collector's curves (c) for Doñana's aquifer samples 49S1 and S56. Rarefaction curves and Chao1 richness estimate collector's curves were calculated for OTUs defined as groups of sequences with > 97% identity (species level).

cells observed in S56 samples appeared to be relatively bigger and therefore constitute more biomass in comparison with those from 49S1 (Table 7.1). However, since the standard deviations for these values were high, this observation would need further confirmation. At any rate, the average cell size was of 0.8-1 μm^3 for both aquifer samples, which is in the range of cell volumes

reported in the literature for other aquifer samples (0.03 to 1.14 μm^3; *e.g.*, Balkwill and Ghiorse, 1985; Bernard *et al*., 2000; Griebler *et al*., 2002). This is not surprising, since the size of microorganisms inhabiting groundwater is usually small including, notably, a large diversity of organisms that pass through 0.2-μm-pore filters (Miyoshi *et al*., 2005). In order to identify the microbial lineages that might be responsible for the biological activities measured in the two aquifer samples, we subsequently carried out a molecular survey of the prokaryotic components of the aquifer's microbial communities.

Overall phylogenetic diversity and diversity estimates

We purified nucleic acids from 49S1 and S56 samples, and then amplified and cloned 16S rRNA genes using different combinations of archaeal- and bacterial-specific primers (Materials and Methods). The different 16S rRNA gene libraries were screened and a total of 201 partial sequences generated (71 and 90 bacterial sequences, and 19 and 21 archaeal sequences from 49S1 and S56, respectively). We compared them by BLAST to sequences deposited in GenBank to have a first insight on the phylogenetic diversity present in our samples (Figure 7.1). Subsequently, 75 representative clones were completely sequenced in order to carry out detailed phylogenetic analyses (see below). The bacterial diversity was greater in 49S1 than in S56 libraries, with members of up to nine different bacterial phyla or candidate divisions and a few very divergent phylotypes of uncertain affiliation (Figure 7.1). Taxa identified in 49S1 included the *Proteobacteria*, with representatives of the A*lpha*, *Beta*, *Gamma* and *Delta* subdivisions, *Planctomycetes, Acidobacteria, Nitrospira*e and the candidate divisions or phyla 'Endomicrobia', SPAM, OP11, OP3, and a novel division-level group of bacteria, some of whose members appear to be denitrifying microorganisms forming syntrophic consortia with anaerobic methane oxidising archaea (Raghoebarsing *et al*., 2006). The 'Endomicrobia' is a candidate phylum corresponding to the candidate division Termite Group 1, whose members have been retrieved mostly from termite guts (Stingl *et al*., 2005). By contrast, only members of two bacterial phyla, *Proteobacteria* and *Firmicutes*, were amplified from the S56 sample. In both aquifer samples, the dominant group in libraries was that of the *Betaproteobacteria*, which is frequently and abundantly detected in groundwater (Miyoshi *et al*., 2005; Santoro *et al*., 2006), although the *Gammaproteobacteria* were also very abundant in the S56 library. Despite the fact that the phylum-level bacterial diversity appeared greater in the shallower 49S1, the archaeal diversity appeared wider in S56 (see below).

To have an insight on the degree of exploration of the 16S rRNA gene libraries constructed from the two aquifer samples, we made rarefaction curves and estimated different diversity indexes. We produced two independent alignments, corresponding to the complete sets of partial sequences retrieved from 49S1 and S56, respectively. We then generated the respective distance matrices to produce accurate estimates of the level of phylotype redundancy (rarefaction curves) and to calculate various diversity indices based on the proportional number of species (Materials and Methods). As a practical criterion to define prokaryotic species or, rather, an Operational Taxonomic Unit (OTU), we considered those sequences having more than 97% identity as members of the same OTU (Schloss and Handelsman, 2005). Rarefaction curves for 49S1 and S56 did not

reach saturation, although S56 was much closer to it as the curve seemed to approach an asymptote (Figure 7.2a). In the lineage-through-time plots (Schloss and Handelsman, 2005), the 49S1 curve was also above that of S56, indicating that there were consistently higher numbers of 49S1 than S56 OTUs at different phylogenetic distances (Figure 7.2b). The greater diversity in 49S1 libraries was also reflected by higher values in all the diversity estimates done (Table 7.2). Thus, the Shannon index, widely used to compare OTU richness in different samples, was relatively high in 49S1 at the species level, being moderate in S56. This trend was also observed when considering the genus-level approximation (95% sequence identity). However, it is difficult to compare with other environmental studies of aquifers, since diversity measurements are not usually estimated. We also represented the evolution of the non-parametric species richness estimators Chao1 and ACE as a function of the number of sequences sampled (Figure 7.2c). Both indices yielded similar OTU estimations and, again, estimates were higher for 49S1 than for S56. In addition, we constructed a square distance matrix including the sequences from the two libraries in order to see whether they were significantly different. The comparison of the homologous (C_{49S1}) versus the heterologous ($C_{49S1/S56}$) coverage (Singleton *et al*., 2001) showed that the two sets of libraries were indeed significantly different ($\Delta C_{49S1/S56} = 0.002$, $p = 0.001$).

Archaeal community composition

We detected members of both archaeal kingdoms, *Crenarchaeota* and *Euryarchaeota*, in Doñana's aquifer. However, they appeared to be distinctly distributed, with members of the *Crenarchaeota* only detected in the shallower 49S1 sample and members of the *Euryarchaeota* exclusively in the deeper S56 sample. From the 40 partial sequences analysed, we completely sequenced 12 clones representative of the archaeal diversity found. All the crenarchaeotal phylotypes belonged to the mesophilic Group I, which is largely distributed in marine, freshwater and soil samples. Most of our phylotypes clustered with environmental sequences retrieved from soil and from deep aquifers or mines (Figure 7.3) (Takai *et al*., 2001; Kimura *et al*., 2005), with the exception of Doñana 49S-2A-3, whose closest neighbour was a sequence retrieved from 770-m deep plankton in the Pacific HOT station (DeLong *et al*., 2006). The lifestyle of Group I *Crenarchaeota* was elusive for a long time, although based on the incorporation of labelled bicarbonate during *in situ* incubation experiments, an autotrophic metabolism had been postulated (Wuchter *et al*., 2003). The discovery of *amo*-related genes in genome fragments of soil C*renarchaeota* led to the suspicion that they could oxidise ammonia (Venter *et al*., 2004; Francis *et al*., 2005; Schleper *et al*., 2005; Treusch *et al*., 2005) and, recently, this was confirmed by the isolation of *Nitrosopumilus maritimus*, an autotrophic ammonia-oxidising crenarchaeote (Konneke *et al*., 2005). That the rest of Group I *Crenarchaeota* display also this type of metabolism cannot be assured with certainty. Nevertheless, the fact that various metagenomic crenarchaeote fragments contain *amo*-related genes suggests that this may be the case and that a large proportion of these archaea do play a key role in the nitrogen cycle (Nicol and Schleper, 2006).

Although the bacterial diversity in S56 was relatively limited in terms of detected phyla and that the only archaea found were *Euryarchaeota*, these were highly diverse. S56 archaeal

phylotypes were scattered in the euryarchaeotal tree, with phylotypes belonging to well-defined groups such as the *Methanomicrobiales* and the *Methanosarcinales*, but also to groups of environmental sequences (Figure 7.3). In all cases, our phylotypes had as closest relatives sequences retrieved from environments highly reduced or with strong redox gradients, such as mud volcanoes, hydrothermal sediments, cold seeps or sulphide springs (Figure 7.3). Some of them were identified in hydrocarbon- and chlorinated-solvent contaminated aquifers (Dojka *et al*., 1998). The metabolic capacities of the *Thermoplasmatales*-related and other divergent euryarchaeotal lineages cannot be inferred at present. By contrast, the phylotype Doñana S56-3A-18, well nested within the *Methanomicrobiales*, is most likely a methanogen, since all cultivated *Methanomicrobiales* are methanogens. Similarly, we can reasonably infer the metabolism of the phylotypes belonging to the *Methanosarcinales*. Although many *Methanosarcinales* are methanogens, members of various clusters forming the large ANME2 group are known to be methanotrophic, possibly by performing reverse methanogenesis (Hallam *et al*., 2004), in syntrophy with sulphate-reducing bacteria (Boetius *et al*., 2000, Orphan *et al*., 2001a). Our phylotypes Doñana S56-3A-36 and Doñana S56-3A-21, the most represented euryarchaeotal clone in our library, were very closely related to other ANME2 members, strongly suggesting that they might be anaerobic methanotrophs.

General bacterial diversity

The bacterial diversity was greater in 16S rRNA gene libraries from the shallowest Doñana's aquifer sample, 49S1. The 71 partial 49S1 sequences distributed in various phyla and candidate divisions, whereas from the 90 partial S56 sequences only one belonged to a phylum different from the Proteobacteria, the *Firmicutes* (Figure 7.1). Clones representative of the Proteobacteria (see following section) and the rest of detected bacterial phyla or candidate divisions (Figure 7.4) were fully sequenced for detailed phylogenetic analyses. Phylotypes belonging to phyla other than the *Proteobacteria* and the *Firmicutes* were only identified in 49S1. Some clones affiliated to phyla with cultivated representatives, namely the *Planctomycetes*, the *Acidobacteria* and the *Nitrospirae*, but most of them belonged to candidate divisions without cultivated members (Figure 7.1). *Planctomycetes*-related sequences from Doñana had as closest relative the uncultured anaerobic ammonium-oxidising bacterium *Kuenenia stuttgartiensis*. As revealed from a recent metagenomic analysis, *K. stuttgartiensis* appears highly versatile with respect to energy metabolism (Strous *et al*., 2006). The presence of related sequences in Doñana's pristine aquifer might indicate the occurrence of anammox bacteria, which are key players in nitrogen cycling, in suboxic subsurface environments. We also detected a phylotype (49S1-2B-27) closely related to a sequence from a deep igneous aquifer that belonged to the *Nitrospirae*, a phylum including the nitrite-oxidising genus *Nitrospira* (Figure 7.4). Although the *Nitrospirae* include metabolically diverse bacteria, nitrite oxidation is widespread in this group, and Doñana 49S1-2B-27 appears indeed related to environmental lineages of nitrite-oxidising *Nitrospirae* (Daims *et al*., 2001), although this metabolic activity cannot be directly inferred. The other bacterial phylum with cultivated species having representatives in Doñana's aquifer was that of the *Acidobacteria*. The *Acidobacteria* is a diverse taxon that, so far, has been predominantly detected in soils (Quaiser *et al*., 2003). In fact,

the two different phylotypes identified in the sample 49S1 were most closely related to soil and rhizosphere clones (Figure 7.4).

Table 7.2 Prokaryotic species richness estimates at different levels of Operational Taxonomic Units (OTUs) sequence identity in 49S1 and S56 samples from the coastal Doñana's aquifer. When applicable, 5% confidence intervals (CI) are given.

	49S1 (11-14 m depth)		S56 (74-80 m depth)	
Total Nº of sequences	94		111	
Nº unique sequences	78		76	
Diversity indices	**97% identity ± CI (5%)**	**95% identity ± CI (5%)**	**97% identity ± CI (5%)**	**95% identity ± CI (5%)**
Simpson	0.06	0.07	0.13	0.14
Shannon	3.13 ± 0.23	2.99 ± 0.22	2.29 ± 0.20	2.23 ± 0.23
ACE	64 ± 29	64 ± 27	47 ± 32	50 ± 43
Chao1	56 ± 25	49 ± 26	29 ± 18	30 ± 23

In addition to phylotypes ascribing to known phyla, several Doñana sequences clustered with candidate bacterial divisions and a few divergent ones did not affiliate with any described taxa. This was the case of phylotypes Doñana 49S1-2B-10, closely related to a rhizosphere environmental sequence and potentially related to the *Chloroflexi*, and Doñana 49S1-1B-52, forming a cluster with sequences from deep-sea sediment and crustal fluid (Figure 7.4). The fact that these sequences cluster with other environmental sequences suggests that they do correspond to actual groups of organisms that await description. Some phylotypes ascribed to the Candidate Division OP3, originally identified in a Yellowstone hot spring (Hugenholtz *et al*., 1998) and SPAM, identified in alpine soil (Lipson and Schmidt, 2004). The sequence Doñana 49S1-2B-60 nested within the Candidate Division Termite Group 1, also known as candidate phylum 'Endomicrobia', which are typical endosymbionts of strict anaerobic flagellate protists living in termite guts (Stingl *et al*., 2005). Endosymbiotic 'Endomicrobia' are thought to ferment and deliver hydrogen to the hydrogenosomes of anaerobic flagellates. Nevertheless, 'Endomicrobia' phylotypes are not exclusive of termite guts, since several environmental sequences retrieved from suboxic or anoxic environments belong to this group as well (Figure 7.4). As a matter of fact, the closest relative to Doñana 49S1-2B-60 is a sequence from an australian aquifer (Kimura *et al*., 2005).

OP11 and a novel division-level group identified recently in symbiotic consortia (Raghoebarsing *et al*., 2006) were the two candidate divisions more relatively abundant in Doñana 49S1 libraries. Phylotypes belonging to the OP11 were broadly distributed within this group (Figure 7.4). Candidate Division OP11 was initially identified in a Yellowstone hot spring (Obsidian Pool) together with other OP lineages (Hugenholtz *et al*., 1998). Later, environmental sequences belonging to this large and highly diverse cluster have been found in many different environments having in common the property of being anoxic, reduced and with high concentration of sulphur species (Harris *et al*., 2004). In particular, OP11 appears to be abundant and diverse in deep

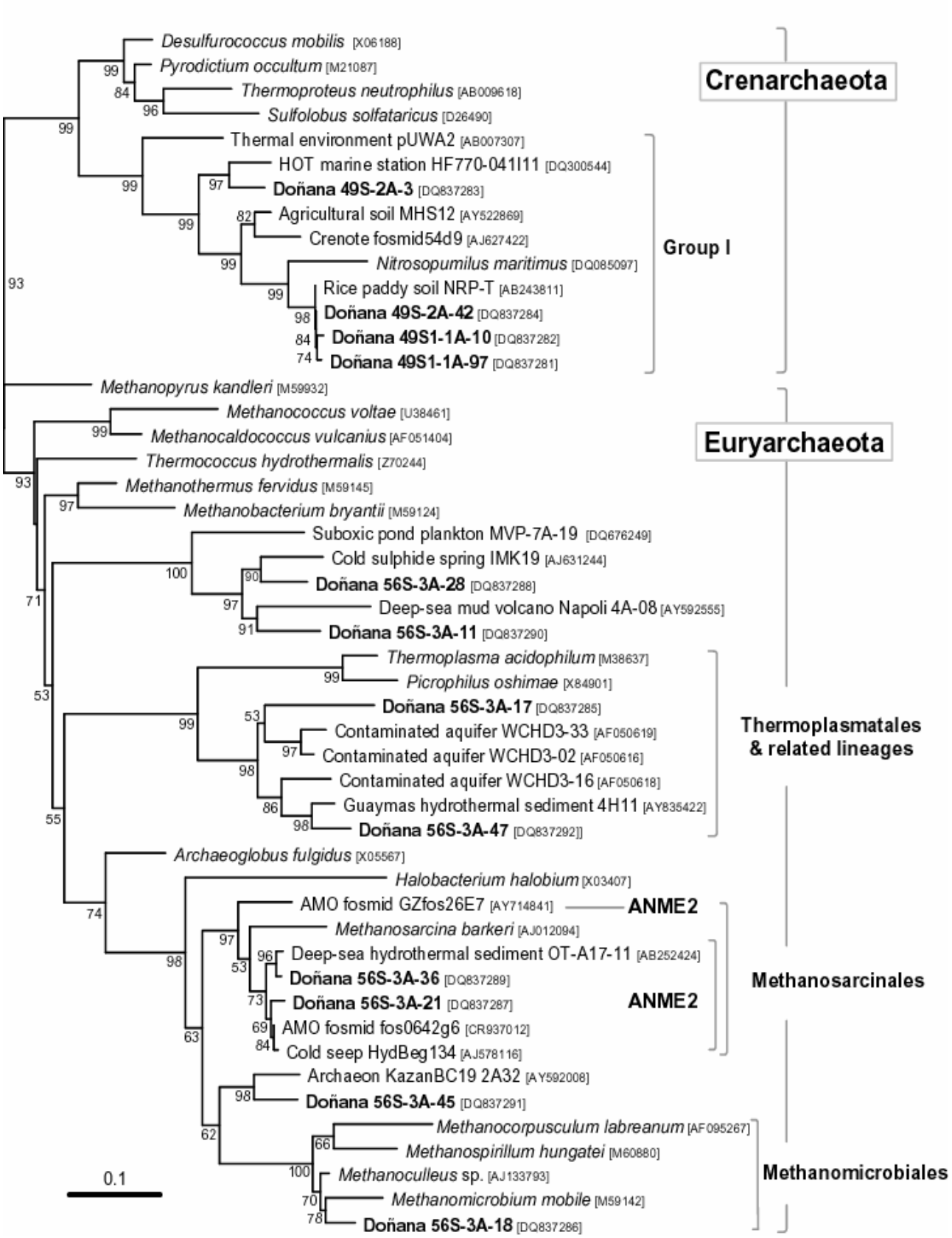

Figure 7.3 Maximum likelihood phylogenetic tree showing the position of archaeal 16S rRNA phylotypes retrieved from a coastal aquifer in Doñana National Park. Italic names correspond to cultivated species or strains, the rest correspond to amplified 16S rRNA gene sequences unless otherwise specified (fosmid). Names in bold correspond to clones obtained in this study. Accession numbers are given in brackets only for environmental clones or non-described species. Only bootstrap values higher than 50% are given at nodes (AOM, anaerobic oxidation of methane).

groundwater, being likely indigenous to subsurface biotopes (Dojka *et al*., 1998; Miyoshi *et al*.,

2005). Although their metabolism is not yet known, given the type of environments where members of this group are found, it might be related to redox reactions involving sulphur compounds (Harris *et al*., 2004). Finally, a relatively high proportion of Doñana bacterial phylotypes was very closely related to the groundwater environmental sequence 12-48 and to the clone D-BACT (Figure 7.4). The latter corresponds to the bacterial symbiont of an archaeal-bacterial consortium mediating the anaerobic oxidation of methane (AOM) coupled to nitrate reduction (Raghoebarsing *et al*., 2006). Until very recently, only AOM consortia coupling methane oxidation to sulphate reduction had been described (Boetius *et al*., 2000; Orphan *et al*., 2001b). These involve syntrophy between ANME1 and ANME2-related *Euryarchaeota* and sulphate-reducing *Deltaproteobacteria*. However, Raghoebarsing *et al*. (2006) succeeded to enrich AOM consortia from anoxic sediments in which putative methanotrophic archaea establish a syntrophic relationship with divergent denitrifying bacteria that form a novel division-level group. Since our Doñana sequences are very closely related to these bacteria, it could be hypothesised that they are denitrifiers that, perhaps, intervene in the AOM reactions. However, the latter point remains speculative, since we failed to amplify euryarchaeotal sequences from the 49S1 sample and, although the archaeal lineages detected by Raghoebarsing *et al*. (2006) in their AOM consortia do not affiliate with classical ANME groups, they are related to *Euryarchaeota* from contaminated soils and ferromanganese nodules in freshwater sediments (Raghoebarsing *et al*., 2006). We cannot exclude that *Euryarchaeota* are indeed present in this part of the aquifer since, given the phylogenetic divergence within archaea, there are no truly archaea-specific primers able to retrieve all archaeal lineages. Nevertheless, it might also be possible that these novel division bacteria are denitrifiers that can or not establish symbiotic consortia to carry out AOM.

Proteobacteria

As mentioned before, both the 49S1 and S56 libraries were dominated by phylotypes ascribed to the *Betaproteobacteria* and, to a lesser extent, the *Gammaproteobacteria* (Figure 7.1). Phylotypes belonging to other proteobacterial subdivisions were much less abundant. Thus, deltaproteobacterial phylotypes were found only in the 49S1 sample. They formed a compact, but quite diverse, group related to sulphate-reducing species of the order *Desulfovibrionales* and one phylotype from Yellowstone's obsidian pool (Figure 7.5a). We indeed detected sulphate-reduction activity in the 49S1 aquifer using the BART^{TM} tests (see materials and methods), indicating that sulphate reduction is at least a potential activity *in situ*. Nevertheless, this method reported a higher sulphate-reducing activity in the S56 samples, despite the fact that we did not retrieve any *Deltaproteobacteria* or other typical sulphate-reducers in the corresponding 16S rRNA gene library. This suggested that sulphate reduction in the S56 aquifer sample was carried out by deltaproteobacterial species that were not recognised by our primers or by organisms not belonging to this phylogenetic group. At any rate, activities measured in the BART^{TM} tests only indicate that those activities may occur *in situ*, as the experimental conditions are different in the laboratory test tubes. The other proteobacterial subdivision found, *Alphaproteobacteria*, was mostly retrieved in 49S1. The only alphaproteobacterial phylotype found S56 was very closely related to a methyl-halide oxidising bacterium belonging to the *Rhodobacteraceae*, which are common aquifer

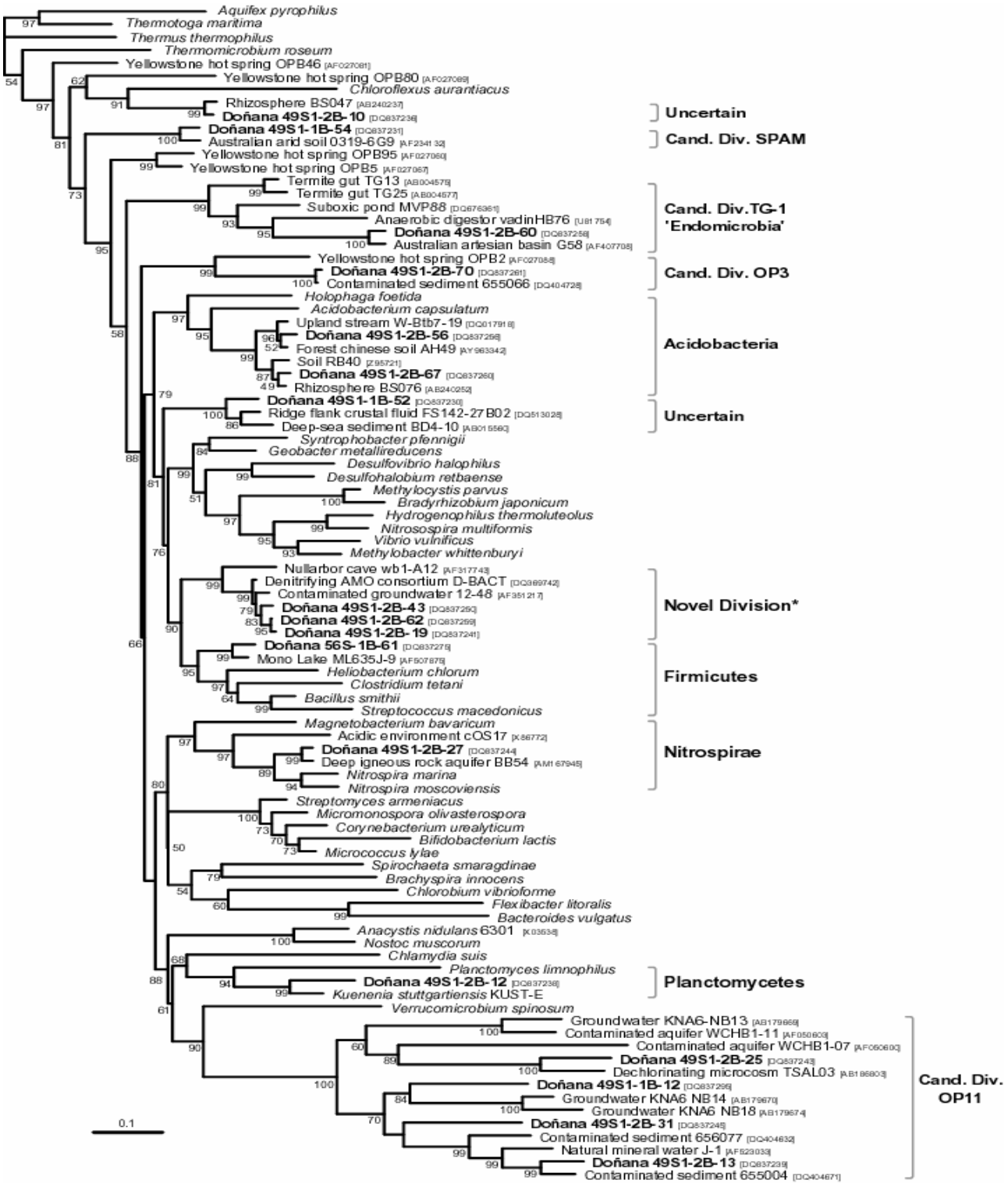

Figure 7.4 Maximum likelihood phylogenetic tree showing the position of bacterial phylotypes retrieved from a coastal freshwater aquifer in Doñana National Park to the exclusion of proteobacterial clones. Italic names correspond to cultivated species or strains, the rest correspond to 16S rRNA environmental clones. Names in bold correspond to clones obtained in this study. Accession numbers are given in brackets only for environmental clones or non-described species. Only bootstrap values higher than 50% are given at nodes. The asterisk indicates the novel division-level group proposed by Raghoebarsing *et al.* (2006) including the bacterial symbiont of a denitrifying AOM consortium (Cand. Div., candidate division; TG-1, Termite Group 1; AOM, anaerobic oxidation of methane).

inhabitants (Ball and Crawford, 2006) (Figure 7.5a). Methyl halides are often associated to human

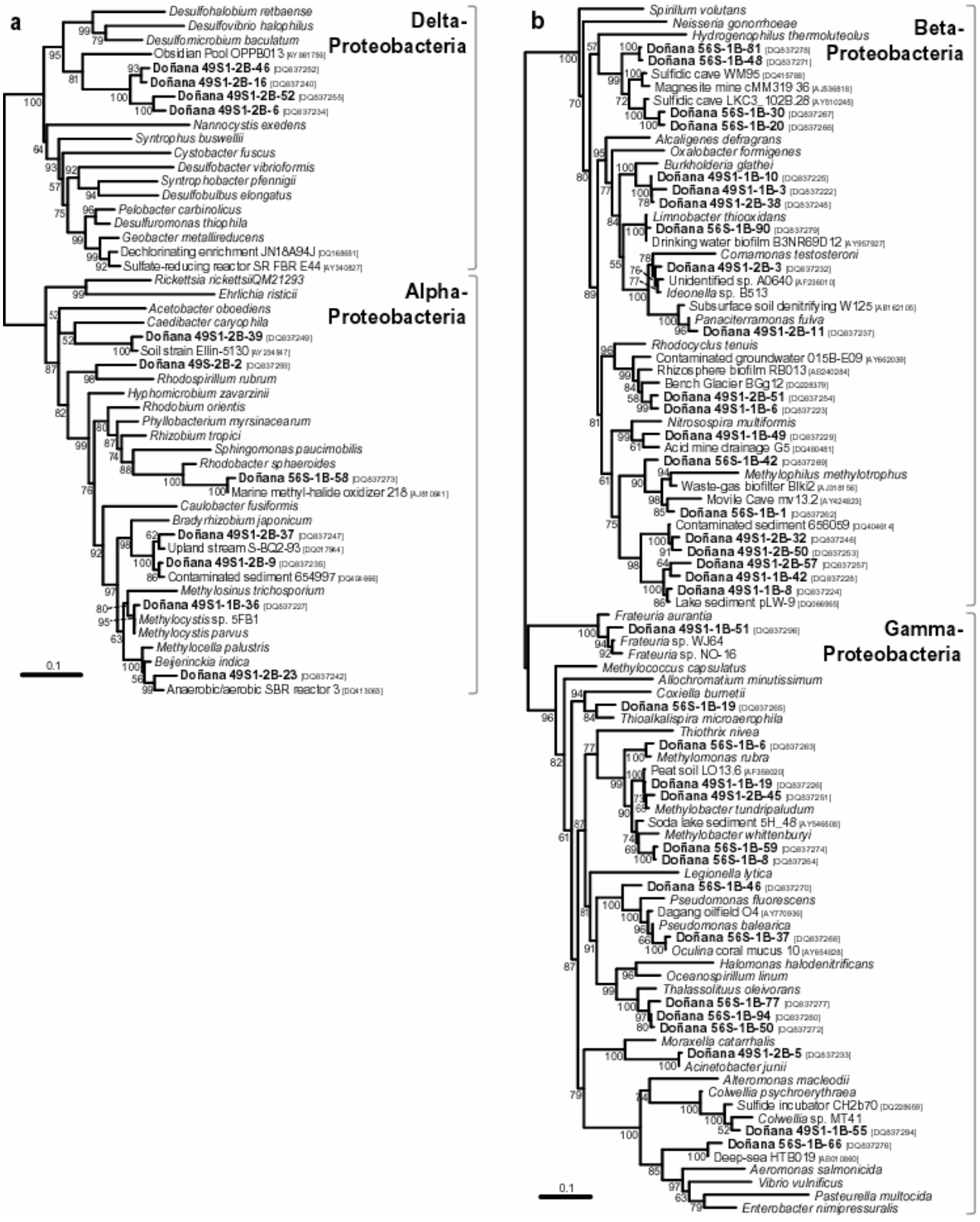

Figure 7.5 Maximum likelihood phylogenetic trees showing the position of *Proteobacteria* phylotypes retrieved from a coastal aquifer in Doñana National Park. Italic names correspond to cultivated species or strains, the rest correspond to 16S rRNA environmental clones. Names in bold correspond to clones obtained in this study. Accession numbers are given in brackets only for environmental clones or non-described species. Only bootstrap values higher than 50% are given at nodes.

pollution, but they can also be present in pristine environments as they are naturally produced,

among others, by certain fungi, plants, marsh communities and during biomass decay (Rhew *et al*., 2003). Some 49S1 alphaproteobacterial phylotypes were closely related to clean- or contaminated-soil isolates, others to aquatic systems, including upland streams and wastewater-treatment reactors. Doñana 49S1-2B-2 had no close relatives, being only distantly related to *Rhodobacter* spp. *Rhodobacter*-related phylotypes have been identified in artesian pristine waters (Ball and Crawford, 2006). By contrast, 49S1-1B-36 was almost identical to several *Methylocystis* spp., including *M. parvus*. These species are aerobic methane-oxidisers, but they can also oxidise a wide range of aliphatic and aromatic compounds (Hanson and Hanson, 1996). Methanotrophic bacteria can also function at very low oxygen concentrations (Roslev and King, 1994), similar to those encountered in Doñana's aquifer samples. As also other gammaproteobacterial methanotroph-like sequences have been found (see below), it could be hypothesised that aerobic methane oxidation might exist in this aquifer under microaerophilic conditions.

Our phylotypes were widely distributed in the betaproteobacterial tree, although there was little overlap between the subgroups found in the two aquifers (Figure 7.5b). A number of S56 phylotypes (Doñana S56-1B-48, S56-1B-81, S56-1B-20, and S56-1B-30) emerged within well-supported groups with other clones retrieved in sulphide-rich environments (caves and mines). These lineages are distantly related to the sulphur-oxidising genus *Thiobacter*, within the order *Hydrogenophilales*. That was also the case for Doñana S56-1B-90, almost identical to the chemolithoheterotroph *Limnobacter thiooxidans*. Most of the 49S1 phylotypes branched within groups containing mostly heterotrophic species, such as those related to the genera *Burkholderia* and *Rhodocyclus* or to the *Comamonas-Variovorax* clade. *Betaproteobacteria* belonging to these groups have been frequently detected in 16S rRNA surveys of aquifers (Pedersen *et al.*, 1996; Ball and Crawford, 2006). The phylotype Doñana 49S1-1B-49 was related to *Nitrosospira*, a genus of ammonium-oxidising bacteria that appears very abundant in anoxic marine sediments (Freitag and Prosser, 2003) and dominates in nitrifying bed reactors (Schramm *et al.*, 1998). Notably, a large proportion of the clones in the S56 library (26 clones nearly identical to Doñana S56-1B-1 in Figure 7.5b) were related to the betaproteobacterial genus *Methylophilus*, together with one phylotype slightly more distantly related. *Methylophilus spp*. metabolise a variety of C1 compounds other than methane (methanol, methylamines, etc.), being present in lake sediments (Nercessian *et al.*, 2005).

We observed similar trends for the gammaproteobacterial phylotypes, since Doñana sequences were also broadly scattered within this group and since clones from the two depth samples were not generally mixed. The only exception was that of gammaproteobacterial methanotrophs, including members of the genera *Methylomonas* and *Methylobacter* (Figure 7.5b). Both, 49S1 and S56 sequences were found to affiliate to this cluster, suggesting that aerobic methanotrophy might potentially occur at both aquifer depths. In addition to methanotrophic-like sequences, most of the remaining gammaproteobacterial phylotypes were closely related to typically heterotrophic genera such as *Frateuria*, *Pseudomonas*, *Thalassolituus*, *Acinetobacter*, and *Colwellia*. Doñana S56-1B-19 was closely related to *Thioalkalispira microaerophila*, a versatile lithoautotrophic oxidiser of sulphur species (Sorokin *et al*., 2002).

As mentioned above, betaproteobacterial phylotypes were by far the most diverse and abundant in 49S1 libraries. They were also very diverse and abundant in S56 libraries but, in this case, they were nearly equalled by the *Gammaproteobacteria* (Figures 7.1 and 7.5). *Betaproteobacteria* are abundantly found in freshwater, including groundwater (*e.g.*, Lindstrom *et al*., 2005; Miyoshi *et al*., 2005; Van der Gucht *et al*., 2005) and, therefore, it is not surprising to find them associated with the Doñana's aquifer. *Gammaproteobacteria* are also widespread in many environments, but are particularly diverse and abundant in seawater (Venter *et al*., 2004; DeLong *et al*., 2006). This, together with the fact that the S56 sample has a conductivity value intermediate between those of fresh and seawater, suggests that this particular region of the aquifer receives some input of marine water and its associated microbiota. Indeed, some of the S56 gammaproteobacterial phylotypes are closely related to typical marine bacteria, such as *Thalassolituus* and *Colwellia* spp. (Figure 7.5).

In summary, the Doñana's aquifer phylotypes belonging to the highly versatile *Proteobacteria* tended to group with heterotrophic lineages, sulphate-reducers (exclusively in the shallower aquifer sample 49S1) and other S-metabolisers (*Limnobacter*, *Thioalkalispira*), ammonium-oxidising bacteria (*Nitrosospira*-like) and, most of all, with C_1-metabolising microorganisms, including typical methanotrophs of the A*lpha* (*Methylocystis*) and *Gamma* (*Methylobacter, Methylomonas*) proteobacterial subdivisions as well as with methylotrophic species of the *Betaproteobacteria* (*Methylophilus*). This suggests that aerobic methane oxidation, under microaerophilic conditions, might be an important process in the two aquifer sub-systems.

CONCLUSIONS

Most environmental sequences retrieved from Doñana's aquifer were related to phylotypes identified in contaminated aquifers and other underground habitats. Since Doñana's aquifer is a pristine environment, this suggests that a portion of the sequences found in contaminated aquifers actually correspond to the indigenous microbiota associated with subsurface systems. In fact, contaminated aquifers are often polluted with hydrocarbon and chlorinated compounds, but these can be also naturally produced during the decomposition and detrital processing of the organic matter (Sanderman and Amundson, 2005). We detected higher phenol oxidase activities in the Doñana deeper sample. Conversely, greater enzymatic activities related to the degradation of the labile pool of organic matter (nucleic acids, proteins and polysaccharides) were observed in the shallower 49S1 sample. This would agree with the sequential degradation of organic matter as a function of depth, with easily degradable organic pools preferentially degraded in upper layers and complex organic matter fractions reaching deeper aquifer regions (Sanderman and Amundson, 2005).

Several of the Doñana's aquifer phylotypes were closely related to cultivated species of known metabolisms, which, together with the biological activities assayed in the laboratory, allows hypothesising the potential occurrence of some metabolic processes in this ecosystem. Many phylotypes were related to typical heterotrophic bacteria, mostly within the *Proteobacteria*, in agreement with various hydrolytic enzymatic activities measured in the laboratory (Table 7.1). Iron

redox activities were measured in Doñana's aquifer samples (Table 7.1). Sulphate reduction was also detected in both aquifer samples in the laboratory (Table 7.1), although the precise identity of potential sulphate-reducers could not be established in the deeper sample. Sulphur-oxidising bacteria could be represented in our samples by a betaproteobacterial cluster within the *Hydrogenophilales*, *Limnobacter*- and *Thioalkalispira*-like phylotypes and, perhaps, the OP11 division (Harris *et al*., 2004). Nitrogen and methane cycles could be also active in Doñana's aquifer. Many phylotypes were related to species involved in nitrogen cycling. They correspond to lineages that might be responsible for the aerobic oxidation of ammonia to nitrite, such as those related to the classical nitrifier genus *Nitrosospira* within the *Betaproteobacteria* (Schramm *et al*., 1998) and, likely, the crenarchaeotal Group I phylotypes (Francis *et al*., 2005; Konneke *et al*., 2005; Nicol and Schleper, 2006). Members of the methanotrophic proteobacteria, well represented in Doñana's aquifer, can also oxidise ammonia using the methane monooxygenase (Hanson and Hanson, 1996). Phylotypes related to nitrite-oxidising lineages within the phylum *Nitrospirae* are also present. In addition, the presence of sequences related to the versatile anammox planctomycete *K. stuttgartensis* suggests a potential for the anaerobic oxidation of ammonium to N_2 using nitrite (Strous *et al*., 1999). On the opposite direction, denitrification was measured in Doñana's samples (Table 7.1). This activity appears to be important in coastal aquifers where the presence of archaeal *amo* genes has been reported (Francis *et al*., 2005; Santoro *et al*., 2006). Potential nitrate-respiring candidates in Doñana might correspond to phylotypes related to the denitrifiers *Thioalkalispira*, *Burkholderia*, *Limnobacter*, the *Comamonas* group, or the novel denitrifying AOM-related division (Raghoebarsing *et al*., 2006). Finally, lineages involved in methane metabolism also occurred in Doñana's aquifer, particularly in the deepest sample. These included typical methanogenic archaea (*Methanomicrobiales*) and a variety of lineages related to aerobic methane oxidisers (*Methylocystis, Methylobacter, Methylomonas*) and anaerobic methane oxidisers (*Methanosarcinales* ANME2 group). This might indicate that aerobic and anaerobic methane oxidation could co-occur in certain regions of the aquifer. So far, anaerobic methane oxidation by ANME-related archaea had been reported nearly exclusively in marine sediments (Orphan *et al*., 2001a; Knittel *et al*., 2005), although a recent study showed that ANME-related anaerobic methanotrophs coexist with bacterial aerobic methanotrophs in a freshwater lake (Eller *et al*., 2005). The co-occurrence of both methane oxidation processes might be more widespread in nature than previously thought, being also the case for subsurface ecosystems.

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CAPÍTULO 8. Discusión general

8. DISCUSIÓN GENERAL

En comparación con los sistemas acuáticos superficiales, los acuíferos suelen describirse desde un punto de vista ecológico como sistemas permanentemente oscuros, muy estables en cuanto a condiciones fisicoquímicas y con una cierta carencia en cuanto a diversidad de recursos energéticos y materiales (Ghiorse y Wilson, 1988; Madsen y Ghiorse, 1993; Griebler y Lueders, 2009). Sin embargo, las aguas subterráneas de Doñana constituyen un sistema en el que se presentan unos niveles relativamente elevados de nutrientes, entre los que se incluyen diferentes formas inorgánicas de nitrógeno, fósforo o hierro, con concentraciones más cercanas a los niveles presentados por sistemas considerados meso- o eutróficos que a los que caracterizan los sistemas oligotróficos (Coleto, 2003; Velasco Ayuso *et al.*, 2009a; Velasco Ayuso *et al.*, 2009b). Aunque en este trabajo no se han determinado concentraciones de carbono orgánico, en estudios anteriores llevados a cabo en el acuífero de Doñana se comprobó, a través de mediciones indirectas, la presencia de importantes concentraciones de materia orgánica (Coleto, 2003). Además, los elevados niveles de β-D-glucosidasa y de fenol oxidasa medidos en este estudio estarían indicando la presencia de fuentes de carbono en el acuífero (Velasco Ayuso *et al*., 2010b). Del mismo modo, las aguas subterráneas de Doñana no presentan condiciones fisicoquímicas constantes a lo largo de un ciclo hidrológico, sino que muestran oscilaciones más o menos marcadas, aunque de menor intensidad (Velasco Ayuso *et al.*, 2009a; Velasco Ayuso *et al.*, 2009b) que las que se han observado en los sistemas acuáticos superficiales a los que se asocian desde un punto de vista hidroecológico (Álvarez, 2002; Coleto, 2003). En este sentido, el acuífero de Doñana se comporta como un sistema dinámico que muestra variabilidad espaciotemporal en sus variables fisicoquímicas, sobre todo en la temperatura y en el oxígeno disuelto, siendo así diferente, al menos en su parte más somera, de otros acuíferos que se consideran muy estables (Vorobyova *et al*., 1997).

La estructura de las comunidades microbianas del acuífero de Doñana ha sido definida en términos de abundancia bacteriana, biomasa celular y biomasa bacteriana (Velasco Ayuso *et al.*, 2009a; Velasco Ayuso *et al.*, 2009b). En líneas generales, los órdenes de magnitud que presentaron estas variables oscilaron considerablemente desde el punto de vista estacional, aunque no difirieron excesivamente de los valores que se han descrito en otros sistemas acuíferos. No obstante, estos valores están más próximos a los que habitualmente caracterizan las poblaciones microbianas bentónicas de los sistemas acuíferos que de los que presentan las poblaciones planctónicas de estos sistemas, que son precisamente las que han sido objeto de estudio en este trabajo. Los valores medios de estas variables fueron menores que los encontrados en los sedimentos de los mantos eólicos de Doñana (Álvarez, 2002).

La función de las comunidades microbianas en las aguas subterráneas de Doñana ha sido estudiada en términos de biomasa microbiana activa, producción bacteriana de carbono, tasa bacteriana de crecimiento, grupos funcionales, actividades enzimáticas extracelulares y diversidad microbiana (López-Archilla *et al*., 2007; Velasco Ayuso *et al.*, 2009a; Velasco Ayuso *et al.*, 2009b; Velasco Ayuso *et al*., 2010a; Velasco Ayuso *et al*., 2010b). Los valores medios estimados en este trabajo para las tres primeras variables fueron, en términos generales, un poco más bajos de los que se han determinado en otros sistemas acuíferos tanto sedimentarios como graníticos. Hay que tener en cuenta que la estacionalidad de los muestreos puede hacer que estos valores medios oscilen más que en el caso de muestreos puntuales, que son los que habitualmente se llevan a cabo en los trabajos que se publican sobres sistemas acuíferos (Velasco Ayuso *et al*., 2010a). La presencia de microorganismos activos sulfatorreductores, desnitrificantes y del hierro ha quedado demostrada gracias al análisis de grupos funcionales mediante el uso de ensayos en tubos BART^{TM} (*Biological Activity Reaction Tests*); los microorganismos del acuífero de Doñana están directamente implicados, por tanto, en los ciclos biogeoquímicos del azufre, del nitrógeno y del hierro (Velasco Ayuso *et al.*, 2009a; Velasco Ayuso *et al.*, 2009b). Las actividades enzimáticas extracelulares (AEE) (β-D-glucosidasa, leucina aminopeptidasa, fosfatasa alcalina y fenol oxidasa) determinadas en las aguas subterráneas de Doñana se encuentran en el mismo orden de magnitud que las estimadas en otros acuíferos, aunque son claramente inferiores a las estimadas en los sedimentos de los humedales hipogénicos de Doñana (Álvarez, 2002); en cualquier caso, la presencia de estas actividades enzimáticas extracelulares demuestra no solamente que las comunidades microbianas del acuífero de Doñana están activas sino que poseen un papel importante en las transformaciones que se llevan a cabo en los ciclos biogeoquímicos del carbono, del nitrógeno y del fósforo. El estudio de la diversidad microbiana del acuífero de Doñana ha sido abordado mediante técnicas de amplificación, clonación y secuenciación de genes de ARN ribosómico 16S (López-Archilla *et al*., 2007), comparando las aguas de dos piezómetros, uno somero (49S1) y otro profundo (S56). La diversidad bacteriana fue más elevada en el piezómetro más somero mientras que la diversidad de arqueas fue mayor en el piezómetro más profundo. Los resultados obtenidos en este estudio permiten afirmar que los microorganismos del acuífero de Doñana están directamente implicados en los ciclos biogeoquímicos del carbono, del azufre y del nitrógeno, lo que refuerza los resultados

obtenidos tras los ensayos en los tubos BART™ y la determinación de las actividades enzimáticas extracelulares.

Los patrones de distribución de los microorganismos en los sistemas acuíferos pueden explicarse en función de diferentes variables dependiendo de las aproximaciones escalares espaciales y temporales a las que se estudien (Madsen y Ghiorse, 1993). A grandes escalas, existen diversos estudios sobre la relación entre estos patrones y variables climatológicas, geomorfológicas, litológicas e hidrológicas. Sin embargo, las variables que determinan la distribución espaciotemporal de las comunidades microbianas en los acuíferos apenas se han estudiado a pequeña escala (Griebler y Lueders, 2009).

Entre las variables climáticas que más suelen influir en estos patrones de distribución en los sistemas naturales se encuentra la temperatura, que suele regular las abundancias y las actividades de las comunidades microbianas (Hoppe *et al.*, 2002). En las aguas subterráneas de Doñana, la temperatura del agua se ha correlacionado directamente con la abundancia bacteriana, la biomasa celular o la biomasa bacteriana en casi todos los piezómetros estudiados, salvo en algunos muy profundos como SO2a, SO2b y SO1. El patrón temporal que ha mostrado la temperatura en las aguas subterráneas es relativamente parecido al de las aguas superficiales, con mayores temperaturas en verano y otoño que en invierno y primavera, aunque con una menor oscilación estacional (Coleto, 2003; Velasco Ayuso *et al*., 2009a; Velasco Ayuso *et al*., 2009b). Pequeños cambios en la temperatura de las aguas subterráneas provocan cambios en la estructura de las comunidades microbianas en términos de abundancia bacteriana, biomasa celular y biomasa bacteriana, sobre todo en la parte más somera del acuífero (Velasco Ayuso *et al*., 2009a; Velasco Ayuso *et al*., 2009b). Por tanto, los resultados obtenidos en este estudio demuestran que la biota microbiana de este acuífero es muy reactiva frente a pequeñas variaciones de la temperatura del agua, lo que contradice en parte la hipótesis general de esta tesis y los resultados obtenidos en otros sistemas acuíferos (Vorobyova *et al*., 1997). Sin embargo, aunque la temperatura influye claramente sobre algunas variables estructurales que definen las comunidades microbianas en el acuífero de Doñana, los resultados obtenidos en relación a otras variables parecen indicar que este factor no es el único que controla la dinámica espaciotemporal de los microorganismos en el acuífero (Velasco Ayuso *et al.*, 2009a; Velasco Ayuso *et al.*, 2009b). Así, pese a que la temperatura determinó notablemente las actividades microbianas estimadas a través de los ensayos en tubos BART™, sus relaciones con otras variables que determinan igualmente la función de las comunidades microbianas en este acuífero (producción bacteriana de carbono o biomasa microbiana activa) son poco claras. Todo lo observado en este trabajo coincide con el hecho generalmente aceptado de que entre las variables que controlan las actividades microbianas se incluyen la disponibilidad de agua y de nutrientes, pero no siempre la temperatura (Harris y Tibbles, 1997). De hecho, Sinsabaugh y Follstad Shah (2010) proponen que la temperatura regula, en líneas generales, el funcionamiento de las comunidades microbianas en términos de actividad enzimática extracelular, pero que es realmente la presencia de nutrientes y de carbono asimilable lo que verdaderamente determina en mayor grado su dinámica espaciotemporal en los sistemas naturales. Del mismo modo, Wilczek *et al*. (2005) demostraron que la temperatura puede influir sobre las

actividades enzimáticas en los sedimentos del río Rin, aunque la presencia de nutrientes y de carbono fácilmente asimilable ejerce un papel mucho más importante.

De entre las variables litológicas más comúnmente estudiadas, el tipo de mineral, el tamaño de grano o la textura pueden determinar las abundancias y las actividades de las comunidades microbianas en los sistemas acuíferos, así como sus dinámicas espaciales y temporales. En las aguas subterráneas de Doñana, el tamaño de grano se ha relacionado con la abundancia bacteriana en algunos piezómetros con materiales muy finos en sus rejillas frente a otros que presentan materiales más gruesos, aunque las diferencias no fueron significativas (Velasco Ayuso *et al.*, 2009b). Sin embargo, el resto de las variables microbiológicas estudiadas, tanto las que definen la estructura como las que definen la función de las comunidades, no se vieron afectadas por el tamaño de grano, el tipo de mineral o la textura. Es importante señalar que estas variables litológicas no varían tanto en el acuífero de Doñana como en otros acuíferos, en los que se ha comprobado que sí pueden afectar a la distribución de las comunidades microbianas (Balkwill y Ghiorse, 1985; Brockman y Murray, 1997; Musslewhite *et al*., 2003). El aparente control del tamaño de grano sobre la abundancia celular en las aguas subterráneas de Doñana podría ser debido más bien al control que ejercen ciertas variables hidrológicas que pueden estar condicionadas por él, como la permeabilidad, la porosidad o la transmisividad. Es decir, un contenido elevado en arcillas no controla directamente la densidad celular en el acuífero, sino que es su influencia sobre la conductividad hidraúlica y los flujos hidrológicos lo que podría explicar mejor las diferencias en los patrones espaciales de distribución de esta abundancia.

De hecho, una de las principales conclusiones a las que ha llegado este trabajo es el importantísimo papel que tienen los flujos hidrológicos en la estructura y en la función de las comunidades microbianas en el acuífero de Doñana. Estos flujos hidrológicos, controlados parcialmente por variables litológicas, como se ha comentado anteriormente, conectan las aguas superficiales y las subterráneas, favoreciendo un intercambio de materiales entre ambos compartimentos del gran ecosistema fluviolitoral de Doñana (GED) y posibilitando un espacio continuo en el que se desarrollan los procesos ecológicos que mantienen el correcto funcionamiento del *hidroecosistema* (Montes *et al*., 1998). La dinámica espaciotemporal de los flujos hidrológicos locales determinó, por ejemplo, el patrón de distribución de la abundancia bacteriana en los piezómetros pw1so y psalsol, y de la producción bacteriana de carbono y de la tasa bacteriana de crecimiento en los piezómetros po1d y psalsol, todos ellos someros, localizados en las cercanías de los humedales hipogénicos de Santa Olalla y Dulce, y con comportamientos hidrogeológicos diferentes en relación a ellas (piezómetros *inflowing* o *outflowing*) (Velasco Ayuso *et al*., 2009a; Velasco Ayuso *et al*., 2010b). Del mismo modo, los flujos hidrológicos locales que transportan materia orgánica y nutrientes, generados en los procesos de producción primaria y de descomposición en las aguas superficiales, controlan la dinámica espaciotemporal de las actividades enzimáticas extracelulares determinadas en este estudio, principalmente β-D-glucosidasa, leucina aminopeptidasa y fenol oxidasa, tal y como se ha demostrado en el piezómetro psalsol, claramente influido por las aguas superficiales de Santa Olalla (Velasco Ayuso *et al*., 2010b). Por otra parte, algunos flujos hidrológicos regionales lavan en sus trayectorias ascendentes capas ricas en materia

orgánica; como consecuencia, transportan carbono orgánico y nutrientes hacia piezómetros algo más profundos (como por ejemplo 40S1, 39S2, 38S1 y 39S1) que presentan altas tasas de producción bacteriana de carbono y elevadas proporciones de biomasa microbiana activa, así como importantes actividades enzimáticas extracelulares, fundamentalmente β-D-glucosidasa y leucina aminopeptidasa (Velasco Ayuso *et al*., 2010a; Velasco Ayuso *et al*., 2010b). Finalmente, también se han descrito en este trabajo relaciones inversas entre las precipitaciones y algunas variables microbiológicas que determinan la estructura de las comunidades microbianas en las aguas subterráneas de Doñana, generándose un descenso en las abundancias bacterianas y en las biomasas bacterianas como consecuencia de procesos de dilución (Velasco Ayuso *et al.*, 2009a; Velasco Ayuso *et al.*, 2009b). En definitiva, la variabilidad espaciotemporal de la mayoría de las variables que determinan la estructura y la función de las comunidades microbianas del acuífero de Doñana está mayoritariamente determinada por la dinámica del agua y de los recursos que ésta transporta, estando ambas totalmente ligadas y controladas por los flujos hidrológicos.

El estudio a pequeña escala de la variabilidad espaciotemporal de las comunidades microbianas en los sistemas subsuperficiales ha sido, como ya se ha comentado anteriormente, mucho menos abordado por ser bastante más complejo tanto desde un punto de vista metodológico como desde uno técnico (Griebler y Lueders, 2009). Sin embargo, el estudio de las actividades enzimáticas extracelulares ayuda a comprender parcialmente la dinámica microbiana espaciotemporal a pequeña escala en cualquier tipo de sistema, dado que éstas reflejan, en parte, la percepción a nivel celular que poseen las comunidades microbianas de los nutrientes. Además, teniendo en cuenta que las actividades enzimáticas extracelulares se relacionan directamente con las tasas de producción bacteriana de las comunidades microbianas (Foreman *et al*., 1998), el estudio de esta *percepción microbiana* de los nutrientes permite también comprender parcialmente la dinámica de la producción de carbono a nivel de comunidad. Sinsabaugh *et al.* (1994) proponen que el estudio de las enzimas extracelulares en comunidades microbianas constituye una aproximación inmejorable para integrar la información de diversos procesos que se llevan a cabo a diferentes niveles, desde el celular hasta el de comunidad. En las aguas subterráneas de Doñana se ha encontrado una relación directa entre la producción bacteriana de carbono y las actividades de varias enzimas extracelulares, así como entre la producción bacteriana de carbono y la distribución de recursos estimada mediante el análisis de las relaciones enzimáticas extracelulares. Ello significa que no existe una limitación concreta en la producción bacteriana de carbono debida a un único nutriente, sino que todos los nutrientes actúan como colimitantes (Velasco Ayuso *et al.*, 2010b), haciendo que la falta de uno de ellos limite la energía empleada en la adquisición de los otros o que su presencia catalice el uso de las enzimas extracelulares para obtener los demás. Estas relaciones enzimáticas no son sin embargo tan sencillas como pueden parecer de antemano y dependen de varios factores que actúan simultáneamente. Sinsabaugh *et al*. (2009) y Sinsabaugh *et al*. (2010) sugieren que las dinámicas espaciales y temporales de las actividades enzimáticas extracelulares pueden ser explicadas a través de las teorías metabólica y estequiométrica, recientemente aplicadas en ecología. En este sentido, los niveles de actividades enzimáticas dependen de cómo sea la proporción de nutrientes en el medio en el que se liberan las enzimas en relación con esa proporción

dentro de las células, por un lado, y de cómo las células capten esos nutrientes en función de sus tasas de crecimiento y de sus capacidades de asimilación, por otro. En cualquier caso, las relaciones enzimáticas, que se describen en el capítulo 6, tienden siempre a ser maximizadas con el objetivo de mantener la funcionalidad de la comunidad microbiana en su conjunto, estimada principalmente en términos de producción bacteriana de carbono. Por todo ello, las actividades enzimáticas extracelulares se pueden considerar como una forma de observar el comportamiento microbiano a un nivel celular, aunque sus resultados reflejen el comportamiento general a nivel comunitario. Sin embargo, a pesar de la clara relación entre la producción bacteriana de carbono y las actividades enzimáticas extracelulares, en este trabajo no se han encontrado relaciones directas entre ninguna de estas dos variables, que describen las comunidades microbianas en términos funcionales, y la abundancia celular (Velasco Ayuso *et al*., 2010a; Velasco Ayuso *et al*., 2010b), probablemente como concecuencia de que no todas las células detectadas mediante técnicas de microscopía están activas. No es sorprendente la ausencia de relaciones directas entre las producciones bacterianas de carbono y las abundancias celulares cuando se emplea la técnica de la incorporación de leucina para determinar el crecimiento celular (Velasco Ayuso *et al*., 2010a). En cualquier caso, no es fácil encontrar respuestas sencillas para las relaciones entre las diferentes variables microbianas, sobre todo en sistemas naturales, donde la variabilidad tanto espacial como temporal es enorme y difícil de abordar, tal y como se ha demostrado en algunos estudios llevados a cabo en varios sistemas acuíferos (Brockman y Murray, 1997; Musslewhite *et al.*, 2003).

Aunque en sistemas profundos se han establecido cadenas tróficas microbianas sencillas que dependen poco o muy poco de los aportes de materiales procedentes de otros sistemas circundantes y que suelen presentar importantes poblaciones de bacterias litoautotrofas (Parkes *et al.*, 1994; Pedersen, 2000), en general, los sistemas acuíferos, sobre todo los arenosos y someros, reciben de los sistemas superficiales aportes de materia orgánica y de nutrientes que resultan fundamentales para la organización de los procesos ecológicos que en ellos se llevan a cabo (Madsen y Ghiorse, 1993; Griebler y Lueders, 2009). De hecho, cuanto más cercanos a la superficie terrestre se sitúen los sistemas acuíferos a la superficie terrestre, no solamente podrán ser más abiertos y variables desde un punto de vista espacial y temporal, sino que dependerán más de los sistemas superficiales para llevar a cabo sus procesos ecológicos. En este sentido, en el modelo de conceptualización de los principales flujos de materia y energía entre los compartimentos del *hidroecosistema* de Doñana, el cual ha servido de base para el desarrollo de este trabajo (Figura 1.3), se puede observar la existencia de relaciones hidroecológicas directas entre los sistemas subsuperficiales y los sistemas superficiales que determinan un transporte de materiales entre el acuífero y los humedales hipogénicos (Álvarez, 2002; Coleto, 2003). Los resultados de esta tesis han permitido, desde un punto de vista ecosistémico, obtener datos relativos a las características estructurales y funcionales de las comunidades microbianas del acuífero, dando contenido al compartimento acuífero del esquema conceptual de flujos de materia y energía de la Figura 1.3. La Figura 8.1 muestra un zum del compartimento acuífero mostrado en la Figura 1.3 e incorpora datos medios sobre diversas variables que resumen la estructura y la función de sus comunidades microbianas. La mayor parte de las transformaciones de materia y de energía que se muestran en este esquema están mediadas

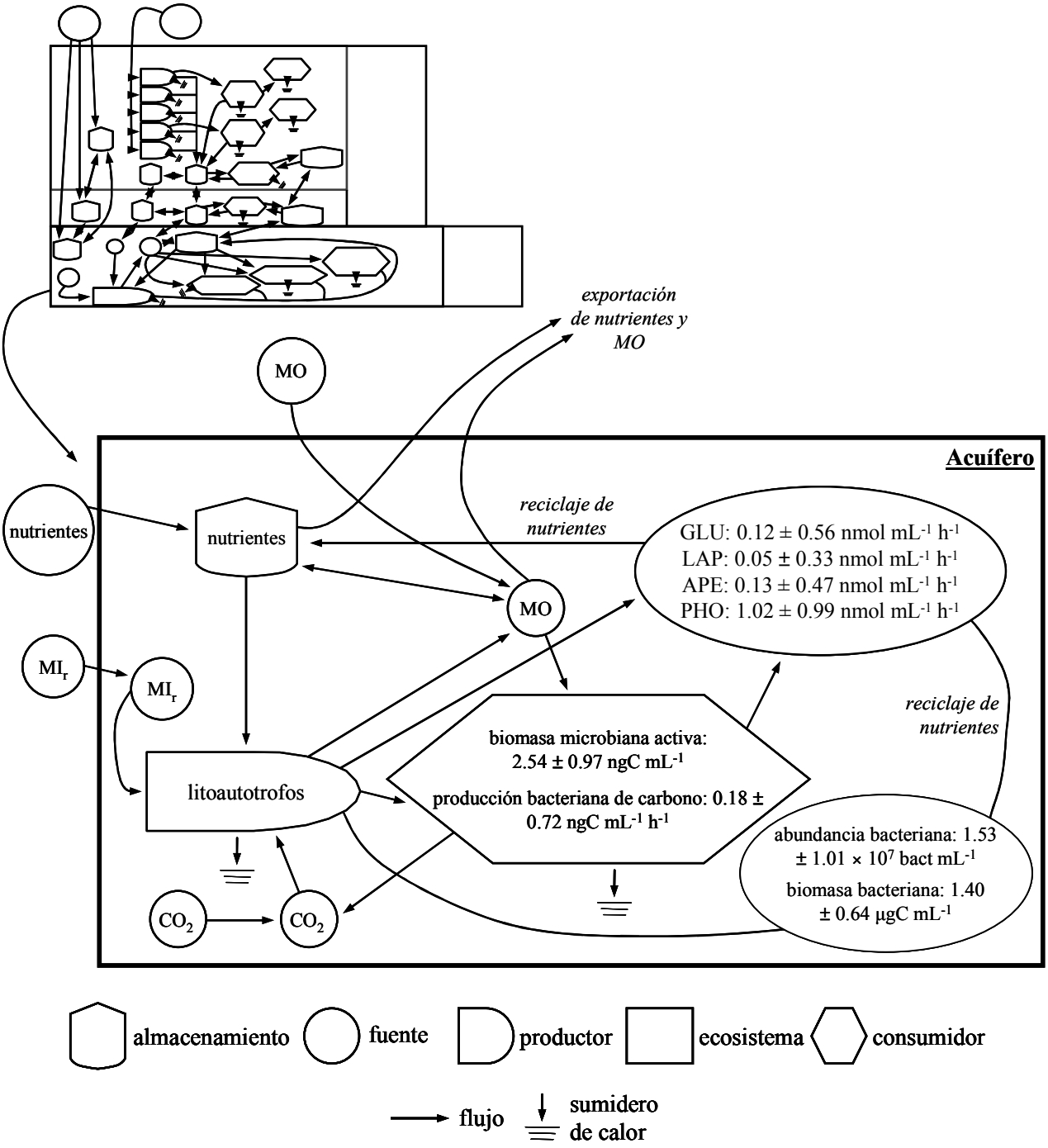

Figura 8.1 Zum del compartimento acuífero del *hidroecosistema* de Doñana en el que se muestran los flujos de materia y de energía siguiendo la nomenclatura de Odum y Barrett (2001). Se incluyen datos medios de diversas variables estimadas en este estudio que resumen la estructura y de la función de sus comunidades microbianas. Se hace especial hincapié en el papel ecológico de éstas como procesadores de materia orgánica y nutrientes, que son exportados hacia otros sistemas, tanto acuáticos como terrestres, gracias a la conexión hidroecológica que existe en el gran ecosistema fluviolitoral de Doñana (GED) (Montes *et al.*, 1998) (MO, materia orgánica; MI_r, materia inorgánica reducida; GLU, β-D-glucosidasa; LAP, leucina aminopeptidasa; APE, fosfatasa alcalina; PHO, fenol oxidasa).

por microorganismos y, la gran mayoría de ellas, exclusivamente por procariotas. Estas comunidades de procariotas actúan como procesadores de materia y de nutrientes, con un importante papel en los procesos de reciclaje, poniendo de nuevo a disposición de las comunidades biológicas de los sistemas superficiales nutrientes y materia orgánica, completando así el proceso de

descomposición iniciado por las comunidades microbianas de los sedimentos de los humedales hipogénicos (Álvarez, 2002).

La presencia de organismos litoautotrofos y organoheterotrofos en las aguas subterráneas de Doñana ha quedado demostrada gracias a los ensayos en tubos BART™ (Velasco Ayuso *et al.*, 2009a; Velasco Ayuso *et al.*, 2009b) y mediante el estudio de la diversidad de procariotas en el acuífero de Doñana a través de la amplificación, clonación y secuenciación de genes del ARN ribosómico 16S (López-Archilla *et al*., 2007). Los resultados obtenidos en estos trabajos han detectado la presencia de microorganismos involucrados en los ciclos biogeoquímicos del azufre, del nitrógeno, del carbono y del hierro. Por otro lado, las actividades enzimáticas extracelulares medidas en las aguas subterráneas de Doñana han puesto de manifiesto el papel de sus comunidades microbianas también en las transformaciones del fósforo (Velasco Ayuso *et al*., 2010b). En general, muchos de los filotipos detectados mediante técnicas moleculares en el acuífero de Doñana están muy relacionados con especies cultivadas y de metabolismo conocido. La mayor parte de estos filotipos están vinculados con bacterias heterotrofas, lo que refuerza la idea de que la presencia de materia orgánica procedente de sistemas superficiales tiene bastante importancia en los procesos ecológicos que se llevan a cabo en el acuífero. También se han identificado filotipos involucrados tanto en la oxidación del azufre como en la reducción de sulfatos. Las actividades sulfatorreductoras fueron detectadas igualmente mediante el uso de ensayos en tubos BART™. El ciclo del nitrógeno está igualmente activo en las aguas subterráneas de Doñana; de hecho, se han encontrado filotipos relacionados con la oxidación del amonio. En el sentido opuesto, los procesos de desnitrificación han sido también detectados en este acuífero mediante técnicas moleculares enfocadas a la determinación de la diversidad procariótica y a través del uso de ensayos en tubos BART™. Algunos de los filotipos presentes en las aguas profundas del acuífero de Doñana están relacionados con microorganismos del metabolismo del metano; entre ellos se incluyen arqueas metanogénicas y una amplia variedad de procariotas metanooxidantes tanto aerobios como anaerobios. La ocurrencia simultánea de ambas rutas metabólicas de oxidación de metano indica que se trata de procesos que se dan en los sistemas naturales con más frecuencia de lo que se pensaba. Ello corrobora la visión actual que se tiene de los acuíferos como un conjunto heterogéneo de microhábitats y macrohábitats que proporcionan una gran cantidad de condiciones diferentes para el desarrollo de las comunidades biológicas, fundamentalmente microbianas (Goldscheider *et al*., 2006). La presencia de microorganismos involucrados en el metabolismo del hierro ha quedado demostrada gracias a los ensayos en tubos BART™. Por tanto, las comunidades microbianas de las aguas subterráneas de Doñana presentan una amplia batería de capacidades metabólicas puestas de manifiesto tanto por técnicas clásicas como por técnicas independientes de cultivo, las cuales se complementan entre sí si se tiene en cuenta que muchos de los microorganismos funcionalmente activos que se encuentran en los sistemas naturales, incluyendo los subsuperficiales, no pueden ser cultivados en laboratorio (Whitman *et al.*, 1998; Rappe y Giovannoni, 2003). Las actividades metabólicas detectadas en las aguas subterráneas de Doñana están implicadas en las transformaciones que tienen lugar en los principales ciclos biogeoquímicos. La Figura 8.2, elaborada a partir de los resultados obtenidos en esta tesis (López-Archilla *et al*., 2007; Velasco Ayuso *et al*., 2009a; Velasco Ayuso *et al*., 2009b;

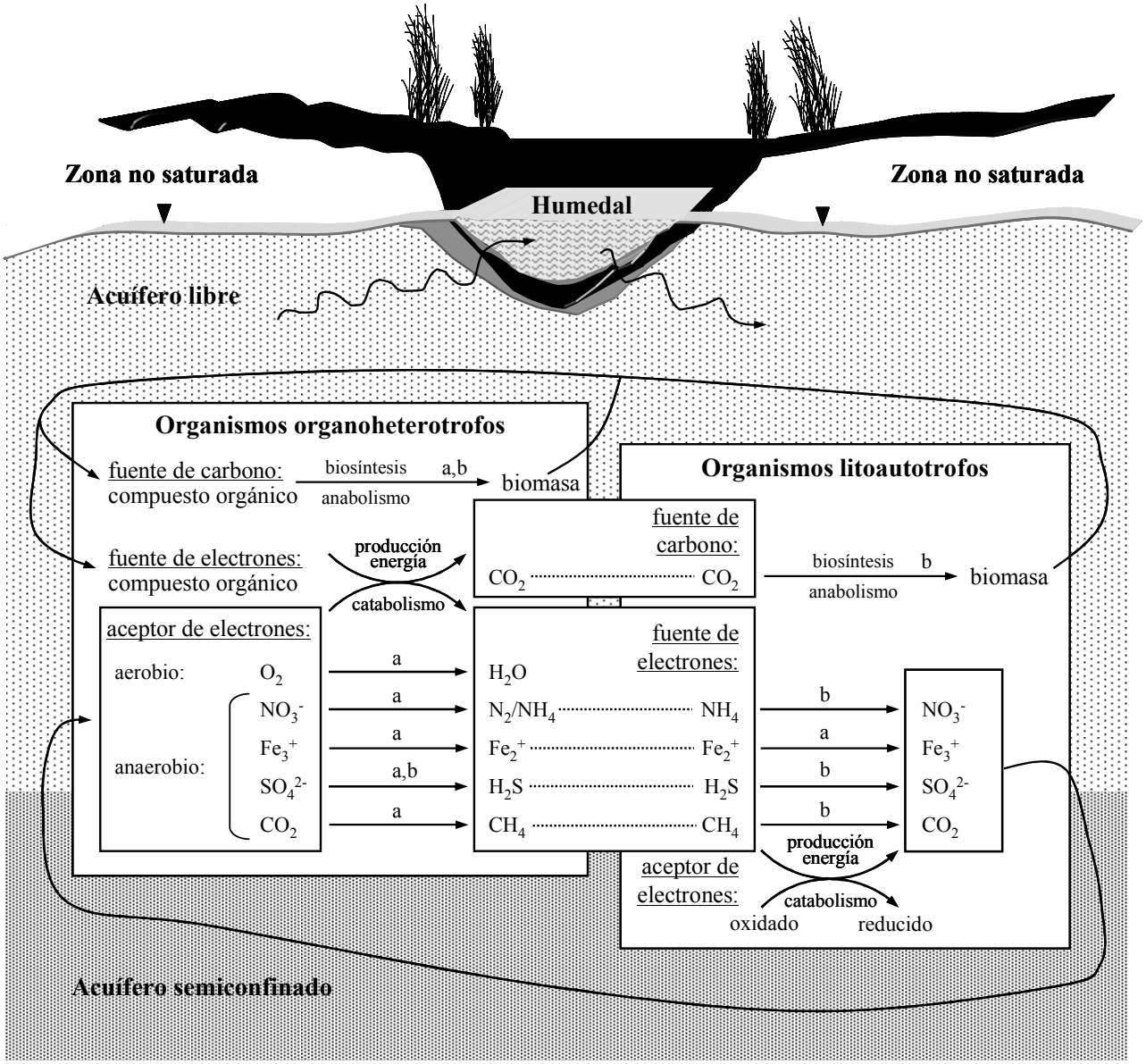

Figura 8.2 Esquema que ilustra los diferentes mecanismos energéticos que pueden estar teniendo lugar en el acuífero de Doñana según las actividades detectadas en los tubos de ensayo BART™ (a) y la información filogenética obtenida a partir de los análisis moleculares de ARN ribosómico 16S (b). Estas rutas metabólicas se llevan a cabo tanto en el acuífero libre como en el acuífero semiconfinado (Basado en Coleto, 2003 y Goldscheider *et al.*, 2006).

Velasco Ayuso *et al*., 2010b), representa un esquema de los diferentes mecanismos energéticos que los microorganismos llevan a cabo en el sistema acuífero, y muestra el papel central que juegan las comunidades microbianas en las transformaciones entre las diferentes formas químicas de los principales nutrientes en las aguas subterráneas de Doñana.

La interpretación conjunta de los datos que se han obtenido en este trabajo, que resulta pionero y abre una nueva línea de investigación en España, permite afirmar que las aguas subterráneas de Doñana no deben ser solamente consideradas como un reservorio de agua ni ser examinadas únicamente desde un punto de vista hidrogeológico, sino que deben ser descritas como un compartimento básico en el funcionamiento ecológico de todo el GED, formando parte del *hidroecosistema* de Doñana (Montes *et al*., 1998). En estas aguas subterráneas se desarrollan unas

comunidades microbianas funcionalmente activas que son capaces de generar y remineralizar materia orgánica, capturar y liberar nutrientes y provocar cambios en los estados de óxido-reducción de diferentes formas químicas. Gracias a la conectividad hidrológica existente entre las aguas subterráneas y las aguas superficiales, se produce un intercambio de estas formas en ambas direcciones, lo que confirma la necesidad de abordar el estudio del *hidroecosistema* de Doñana como un único sistema ecológico a la hora de elaborar planes de gestión de los recursos hídricos. En este sentido, las comunidades microbianas del sistema acuífero de Doñana poseen un papel ecológico muy significativo en el funcionamiento general del GED, de una importancia muy similar a la que tienen las comunidades microbianas que habitan los sedimentos someros de los humedales hipogénicos de Doñana (Álvarez, 2002). Además, los resultados obtenidos en este trabajo apuntan a que el papel ecológico que juegan los microorganismos de las aguas subterráneas en Doñana en relación con los ecosistemas superficiales tiene la misma importancia que el que juegan los microorganismos de las aguas de los medios hiporréicos en relación con los sistemas acuáticos lóticos con los que están conectados (Hancock *et al.*, 2005).

Las aguas subterráneas constituyen, en general, auténticos océanos, aunque fuera de nuestra vista, y ya se sabe lo que dice el refrán, "*si no se ve, no existe*" (Ghiorse y Wilson, 1988). Por tanto, tal y como se ha demostrado en el presente trabajo llevado a cabo en el acuífero de Doñana, muchos acuíferos no deberían ser calificados como áreas semidesérticas colonizadas por comunidades biológicas relícticas y extrañas, sino que deben ser considerados como ecosistemas dinámicos en los que se lleva a cabo una plétora de procesos ecológicos realmente similares a los que pueden observarse en los sistemas superficiales, tanto terrestres como acuáticos.

Con la presente tesis doctoral se ha dado un paso más en el estudio del *hidroecosistema* de Doñana desde un punto de vista ecosistémico, con el objetivo final de obtener las bases científicas de apoyo a la gestión integral de los recursos hídricos de la comarca (Montes *et al*., 1998). Además, gracias a los resultados obtenidos en este trabajo, se puede afirmar que un uso incontrolado del acuífero de Doñana con fines turísticos y agrícolas, no solamente originaría un descenso de los niveles piezométricos, tal y como ya se ha observado (Custodio *et al.*, 2009), sino que también podría provocar una alteración en los flujos hidrológicos subterráneos. Ello traería consigo un cambio en la dinámica funcional de las comunidades microbianas de las aguas subterráneas, lo que podría traducirse en una modificación del papel ecológico que éstas juegan en el *hidroecosistema*. Y una alteración del papel que llevan a cabo los microorganismos en las aguas subterráneas de Doñana podría acarrear cambios muy importantes en otros procesos ecológicos que se desarrollan en los sistemas superficiales, tanto acuáticos como terrestres. Sin embargo, uno de los problemas que dificulta la interpretación de los resultados obtenidos en este trabajo es la distinta escala espaciotemporal a la que se estudian los diferentes procesos (y las variables que los definen) que controlan la estructura y la función de las comunidades microbianas. En la Figura 8.3, elaborada a partir del modelo conceptual de la Figura 1.2, se han introducido las distintas variables que parecen influir en la estructura y en la función de las comunidades microbianas del acuífero de Doñana, mostrando las diferentes escalas a las que habitualmente son estimadas. Por ejemplo, los procesos hidrogeológicos, geomorfológicos y climáticos (y las variables que los determinan) se estudian

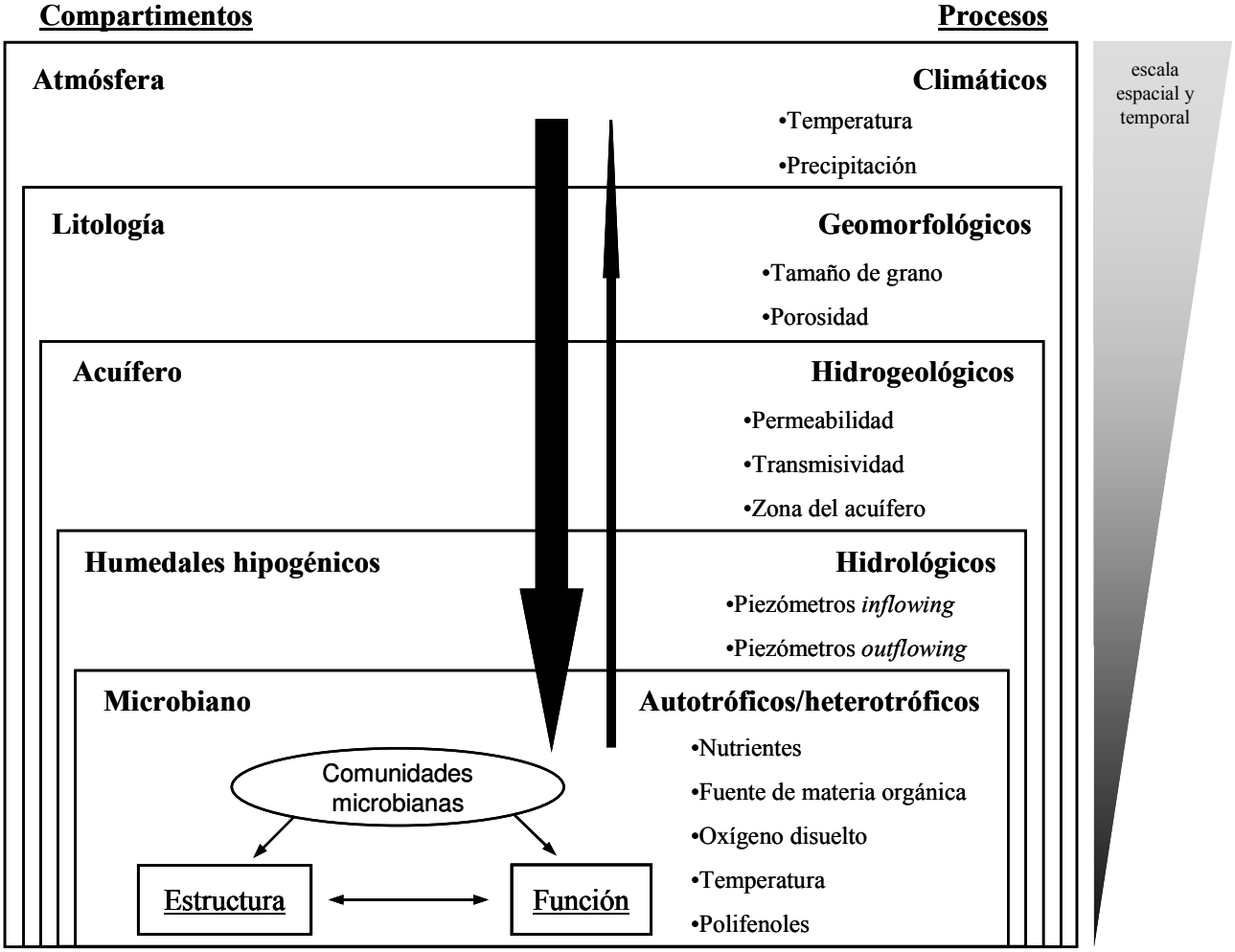

Figura 8.3 Factores que controlan la estructura y la función de las comunidades microbianas del acuífero de Doñana a diferentes escalas espaciales y temporales. Este trabajo demuestra que este tipo de estudios deben realizarse mediante una aproximación ecosistémica basada en un modelo de organización jerárquica de funcionamiento del medio natural, en este caso del *hidroecosistema* de Doñana, que incluye el acuífero, los humedales hipogénicos y las aguas salobres de las marismas (Modificado de Montes *et al.*, 1998).

normalmente a escalas espaciotemporales grandes mientras que los procesos microbiológicos (y las variables que los determinan) se abordan a escalas mucho más pequeñas. Por tanto, para comprender mejor cómo actúan las diferentes variables identificadas en este trabajo sobre la dinámica de las comunidades microbianas en el acuífero de Doñana, sería muy importante que hidrogeólogos y ecólogos microbianos llevaran a cabo aproximaciones conjuntas a la misma escala de trabajo. De esta manera, se podrá obtener una visión más ajustada a la realidad de cómo influyen los flujos hidrológicos y otras variables sobre las comunidades microbianas de las aguas subterráneas, a partir de lo cual se podrán desarrollar modelos de gestión y conservación mucho más efectivos en el espacio natural probablemente más importante de Europa occidental.

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CAPÍTULO 9. Conclusiones

9. CONCLUSIONES

1. La aproximación ecológica al estudio del acuífero de Doñana llevada a cabo durante dos ciclos hidrológicos y en 30 piezómetros de entre 1.8 y 80.0 m de profundidad, distribuidos en un área de 100 km², ha puesto de manifiesto la presencia de comunidades microbianas, compuestas principalmente por bacterias, muy importantes en términos de abundancia bacteriana, biomasa celular y biomasa bacteriana.
2. Las comunidades microbianas del acuífero son funcionales tal y como demuestran los valores de biomasa microbiana activa, producción bacteriana de carbono y tasa bacteriana de crecimiento. Asimismo, los resultados de los ensayos en tubos BART^{TM} indican elevados niveles de actividad de las bacterias del hierro, las bacterias sulfatorreductoras y las bacterias desnitrificantes, mostrando la implicación de estas comunidades en los ciclos biogeoquímicos y en el procesado y reciclaje de materia orgánica y nutrientes.
3. El estudio de la diversidad de procariotas en un piezómetro somero (profundidad de rejilla 11.4-14.2 m) y otro profundo (profundidad de rejilla 74.0-80.0 m), mediante técnicas de amplificación, clonación y secuenciación de genes de ARN ribosómico 16S, muestra la presencia de filotipos relacionados con bacterias quimiolitoautrofas y quimioorganoheterotrofas implicadas en los ciclos del carbono, del nitrógeno y del azufre.
4. La variabilidad espacial de las comunidades microbianas planctónicas del sistema acuífero de Doñana es relativamente baja en términos estructurales, aunque mayor en términos funcionales. La profundidad es un factor que no ejerce ninguna influencia sobre las variables microbianas estudiadas. Otro factor, el tamaño de grano, parece poseer un papel relevante en el control de la abundancia bacteriana, aunque no tanto en el de la biomasa celular. Sin embargo, este factor controla otras variables que pueden determinar la dinámica de los flujos hidrogeológicos (como la permeabilidad, la porosidad y la transmisividad), los cuales sí poseen un papel muy destacable en el control de la variabilidad espacial. Así, en términos de biomasa microbiana

activa, producción bacteriana de carbono y tasa bacteriana de crecimiento, la funcionalidad de las comunidades microbianas está controlada directamente por los flujos hidrológicos debido a que éstos transportan nutrientes y materia orgánica. Como consecuencia, las comunidades microbianas que se localizan en las zonas que reciben mayoritariamente los aportes de estos flujos presentan mayores niveles de actividad.

5. La dinámica temporal observada tanto en las variables estructurales como en la actividad de los grupos funcionales está parcialmente controlada por la temperatura, factor que muestra fluctuaciones estacionales en las aguas subterráneas de Doñana. En este sentido, las comunidades microbianas del acuífero de Doñana presentan una dinámica mucho más parecida a la que poseen las comunidades microbianas de los sedimentos de las lagunas que a la que se ha descrito en las aguas subterráneas de otros sistemas acuíferos. Otras variables que describen funcionalmente las comunidades microbianas del acuífero de Doñana (biomasa microbiana activa, producción bacteriana de carbono y tasa bacteriana de crecimiento) también están controladas por la temperatura, si bien es cierto que el grado de control es mucho menor que en el caso de las variables que definen la estructura de estas comunidades.
6. Las actividades enzimáticas extracelulares determinadas (β-D-glucosidasa –GLU–, leucina aminopeptidasa –LEU–, fosfatasa alcalina –APE– y fenol oxidasa –PHO–) han puesto de manifiesto que la comunidades microbianas del acuífero de Doñana juegan un importante papel en los procesos de degradación, asimilación y reciclaje de materia orgánica y nutrientes.
7. La variabilidad espacial y temporal que presentan las actividades enzimáticas extracelulares estimadas en las aguas subterráneas de Doñana está controlada principalmente por flujos hidrogeológicos, tanto locales como regionales. Estos flujos transportan materia orgánica y nutrientes, dos factores fundamentales que mantienen la funcionalidad de las comunidades microbianas en los sistemas naturales.
8. Las relaciones enzimáticas GLU/LAP y GLU/APE, consideradas como una medida indirecta de la *percepción microbiana* de la disponibilidad de compuestos de nitrógeno y fósforo, varían estacionalmente en relación a los aportes de materia orgánica. La estrategia de distribución de recursos observada para las actividades enzimáticas extracelulares está relacionada con las teorías metabólica y estequiométrica, recientemente aplicadas en ecología.
9. Las relaciones enzimáticas GLU/LAP y GLU/APE han demostrado que el fósforo es el elemento potencialmente más limitante en el funcionamiento de las comunidades microbianas, probablemente porque puede adsorberse a compuestos de hierro, muy abundantes en el acuífero de Doñana, o porque la presencia de polifenoles puede acomplejar la actividad de la fosfatasa alcalina. En este sentido, la presencia de actividad fenol oxidasa puede considerarse como fundamental para facilitar la actividad de las otras enzimas, una vez que los compuestos polifenólicos, potencialmente tóxicos, han quedado degradados. Además, este trabajo constituye el primero a nivel mundial en donde se han determinado actividades de la enzima fenol oxidasa en aguas subterráneas.
10. La aproximación intensiva llevada a cabo durante dos ciclos hidrológicos en 13 piezómetros someros localizados en los alrededores de cuatro lagunas de Doñana bien caracterizadas en

estudios previos, ha demostrado que los patrones espaciotemporales de las comunidades microbianas de los sedimentos de las lagunas y de las aguas subterráneas están controlados por las mismas variables.

11. Las conexiones hidrológicas verticales bidireccionales entre el acuífero y los humedales, previamente descritas, favorecen el transporte de bacterias, materia orgánica y nutrientes desde los sistemas superficiales hacia las aguas subterráneas donde, a través de procesos ecológicos controlados principalmente por microorganismos, las características geoquímicas de las aguas se ven afectadas antes de regresar de nuevo a los sistemas superficiales. Por lo tanto, el sistema acuífero de Doñana no se comporta como un ecosistema aislado, sino más bien como un sistema abierto que intercambia permanentemente, a diferentes escalas espaciales y temporales, materia y energía con los sistemas superficiales formando un ente único, llamado *hidroecosistema*, que funciona como un todo.
12. Considerando conjuntamente los datos de producción bacteriana de carbono, grupos funcionales detectados y actividades enzimáticas, este estudio constituye una evidencia del importante papel que poseen las comunidades microbianas en el procesado de la materia orgánica y en los ciclos biogeoquímicos en las aguas subterráneas de Doñana. Así, los procesos de descomposición que se inician en los sedimentos de los humedales continúan en el acuífero, al menos en su parte más superficial, siendo los materiales posteriormente transportados y puestos a disposición de niveles tróficos superiores gracias a la conexión hidroecológica existente entre los compartimentos superficial y subterráneo del gran ecosistema fluviolitoral de Doñana (GED). Por lo tanto, las comunidades microbianas del acuífero de Doñana poseen un papel ecológico central en el *hidroecosistema* y, con respecto a los ecosistemas superficiales con los que el acuífero se relaciona, similar al que poseen las comunidades microbianas de los medios hiporréicos en relación con los sistemas lóticos.
13. A partir de los datos obtenidos sobre grupos funcionales y sobre diversidad de procariotas, se ha elaborado un modelo conceptual de los diferentes mecanismos energéticos que presentan los microorganismos en el acuífero de Doñana. La diversidad metabólica implica tanto organismos quimioorganoheterotrofos como quimiolitoautotrofos, con actividades complementarias oxidantes y reductoras de distintas formas de carbono, de nitrógeno, de azufre y de hierro, en un gradiente de condiciones variantes de óxicas a anóxicas.
14. Una correcta aproximación a las aguas subterráneas de Doñana desde un punto de vista ecosistémico depende del trabajo conjunto entre ecólogos microbianos e hidrogeólogos. Sin embargo, las escalas de estudio a las que habitualmente se han llevado a cabo los estudios hidrogeológicos son mucho más grandes que las empleadas en los estudios microbiológicos. En estudios futuros, la obtención de una visión más precisa sobre la estructura y la función de las comunidades microbianas de las aguas subterráneas de Doñana, así como de los factores que las controlan, pasa irremediablemente por una aproximación conjunta de ambas disciplinas a las mismas escalas espaciales y temporales.